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The Intracellular Mode of Action of the Sulphonamide Derivatives. Some Condensation Products of Reductone

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Colebrook & Kenny (1936), Colebrook, Buttle & O'Meara (1936) and, later, others showed that some time must elapse after bacteria are inoculated into media containing sulphonamide before an inhibitory effect is produced. Multiplication of the organism occurs in the interval. Subsequently, Wolff & Julius (1939) concluded that sulphonamides act on bacteria only when they are multiplying, and O'Meara (1942) mentioned that the lethal action of sulphonamides on bacteria is immediately exhibited when the organisms are in the logarithmic phase of growth. This was later confirmed and fully expounded by O'Meara, McNally & Nelson (1947), who pointed out that in the logarithmic phase of growth bacteria are engaged in the utilization of those compounds, present in the culture medium, which are most suited to energy and growth requirements. Glucose is one of the substances. O'Meara, McNally & Nelson (1944) had previously noted that during the logarithmic phase of growth the medium develops strongly reducing properties, and suggested that the reducing substance might be either ascorbic acid, dihydroxyacetone or reductone,

$$CH_2OH.CO.CHO \leftrightharpoons CH(OH):C(OH).CHO,$$

all of which could be produced from glucose. In the earlier publication, O'Meara *et al.* (1944) showed that the reducing substance gave a positive 'enediol' [—C(OH):C(OH)—] test with *o*-dinitrobenzene and sodium hydroxide (Fearon & Kawerau, 1943). Ascorbic acid is known to be produced in certain bacterial cultures (Büsing & Peters, 1940), but this can usually be detected as its dehydro derivative (Fearon & Kawerau, 1943) and the reducing substance produced by the pathogens failed to give the test for dehydroascorbic acid. Reductone is produced when glucose is heated with alkali (von Euler & Klussmann, 1933; von Euler & Martius, 1933) and, since the culture media are alkaline and gave the enediol reaction after heating, O'Meara *et al.* (1947) considered that reductone was the most likely substance to be formed in such media.

O'Meara *et al.* (1947) showed that reductone is readily condensed with *p*-aminobenzoic acid, sulphapyridine, sulphathiazole and sulphanilamide. The reductone used was a crude solution from the hydrolysis of glucose with sodium hydroxide, nevertheless solid products were obtained in all cases except in the reductone-sulphanilamide condensation, where the product was obviously readily soluble in water. The products were hydrolysed in alkaline solution with varying degrees of rapidity, yielding the starting materials. Condensation of the above-mentioned amines with ascorbic acid and dihydroxyacetone gave coloured solutions only.

In view of the known biological significance of *p*-aminobenzoic acid, and the fact that the compound formed by union of reductone with *p*-aminobenzoic acid is readily hydrolysed within the biological range of pH, O'Meara *et al.* (1944, 1947) considered that the function of *p*-aminobenzoic acid in cellular metabolism is to stabilize and temporarily conserve, for use of the cell, the extremely active substance reductone which can act as a source of energy for the growing cell. Since reductone is readily oxidized (compare von Euler & Martius, 1933) it must be preserved in a stabilized form, but in such a way as to be available for the requirements of the growing cell. It was, in fact, shown by O'Meara *et al.* (1947) that *Streptococcus pyogenes* can utilize the reductone-*p*-aminobenzoic acid condensation product and maintain growth on it, but *p*-aminobenzoic acid itself fails to support growth. In extension of the above, it was logical for O'Meara *et al.* (1947) to consider that sulphonamides interfere with this source of energy by combination with reductone to form compounds not available for utilization by bacteria. They showed that *Strep. pyogenes* was unable to utilize the sulphapyridine and sulphathiazole derivatives of reductone under conditions identical with those in which the *p*-aminobenzoic acid derivative was utilized by these organisms.

Reductone possesses a highly active aldehydic group which readily reacts with electron-donating groups (compare von Euler & Martius, 1933) and reaction may take place by end attack on the charged mesomeric form of the enol

$$^{+}CH(OH).C(OH):CHO^{-}.$$

The condensation products of reductone and *p*-aminobenzoic acid and the various sulphonamides

are highly coloured, and it is probable that they are of the type

$$HC(OH):C(OH).CH:N.C_6H_4.R$$

or

$$CH_2(OH).CO.CH:N.C_6H_4.R$$

$$(R = CO_2H \quad \text{or} \quad SO_2NHR').$$

According to the theory propounded by O'Meara *et al.* (1947), the effectiveness of the sulphonamides in interfering with the growth of bacteria is linked with the stability of the Schiff bases mentioned above. Those which are most readily hydrolysed will be the least effective in inhibition of growth and vice versa, and it is very likely that the ease of hydrolysis of the Schiff bases in the biological range of pH will be dependent upon the solubility of these compounds under these conditions. Before a final conclusion can be reached, the relative solubilities of the compounds must be determined, but it is known that sulphanilamide gives a condensation product with reductone which is very readily soluble in water at neutrality and difficult to isolate, and that sulphanilamide is relatively ineffective as growth inhibitor.

In accordance with the views expressed on the importance of reductone, it seems likely that in mixtures of *p*-aminobenzoic acid and a sulphonamide with reductone there will be competition for the aldehydic group by the amino groups. This can probably be represented by the following equation, where only the enolic forms are given,

$$OH.CH:C(OH).CH:N.C_6H_4.COOH + H_2N.R \rightleftharpoons OH.CH:C(OH)CH:N.R + NH_2.C_6H_4.CO_2H$$

$$(R = H_2N.C_6H_4.SO_2NHR').$$

The position of equilibrium in the above reaction will depend, amongst other factors, upon (*a*) the affinity of the aromatic amino groups for the aldehydic group, i.e. upon the relative electron-donating qualities of these groups, and (*b*) upon the concentration of the amino compounds. According to Bell & Roblin (1942) and Albert & Goldacre (1942) the basic dissociation constant of the aromatic amino group in the sulphonamides differs only slightly from $2{\cdot}6 \times 10^{-12}$, which is the basic dissociation constant of *p*-aminobenzoic acid. Maphenide (marfanil, *p*-aminomethylbenzenesulphonamide) is in another category. Here the amino group is aliphatic and the compound correspondingly more basic. In general, it is probable that the concentrations of the amino compounds are important in deciding the position of equilibrium. In the bacterial cell other factors might have prominence such as degree of adsorption of the various compounds on some surface.

In the present investigation our primary object has been the preparation and examination of the products of condensation of reductone with *p*-aminobenzoic acid and various sulphonamides. We have obtained solid products in all cases, but purification, by crystallization, has not always been possible owing to the low solubility of the products in most organic solvents. The reductone-sulphanilic acid condensation product is very readily soluble in water, and we have not been able to isolate this compound although its formation undoubtedly occurs, since the colour of the mixed solutions rapidly becomes a deep red.

Although our investigations are not complete we feel that an interim survey is necessary since Angier, Stokstad, Mowat, Hutchings, Boothe, Waller, Semb, SubbaRow, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith (1948) have recently published details of the preparation and properties of the reductone *p*-aminobenzoic acid condensation product and some of its derivatives, and Forrest & Walker (1948) have also indicated the scope of their work similar to that which we had contemplated (cf. Bell, Cocker & O'Meara, 1948), and that which has been in hand since September 1947 (cf. O'Meara *et al.* 1947).

EXPERIMENTAL

The preparation of reductone. Reductone is best prepared by the method of von Euler & Martius (1933) in which glucose is treated with NaOH and the reductone is precipitated as its Pb salt. No improvement in yield was obtained when fructose was employed. The decomposition of the Pb salt with H_2S was, however, performed with shaking in a previously evacuated flask attached to a source of H_2S. This gave a better product than that obtained when H_2S was bubbled through a suspension of the Pb salt (cf. Kuchlin & Böeseken, 1928). The yield of reductone, m.p. 200°, was about 7% of theory. The oxidation of glycerol by $FeSO_4$ and H_2O_2 (den Otter, 1937) and the oxidation of dihydroxyacetone with copper acetate in ultraviolet light (Kuchlin & Böeseken, 1928) gave disappointing results.

Condensation of reductone with amino compounds. The earlier condensations of reductone with the various amino compounds were performed in aqueous sodium acetate, but it was later found that the condensations could be readily performed in aqueous acetone with or without the addition of a few drops of acetic acid. A better product was thus obtained.

Analysis of compounds formed. The identity of the compounds was established by analysis and by colorimetric estimation of the *p*-aminobenzoic acid or sulphonamide in the compound. This was performed by hydrolysis with dilute alkali, and the resultant amino compound was condensed with *p*-dimethylaminobenzaldehyde (Werner, 1939, 1944), the colour so obtained being examined on the Spekker absorptiometer. It was possible approximately to estimate the reductone-*p*-aminobenzoic acid condensation product by titration with alkali, but the values of the equivalent obtained were variable. When the compound was left in contact with 0·05 N-NaOH, the equivalent was found to be 57–60 in contrast to 110–120 which was obtained by rapid solution of the compound in the alkali, followed by back titration. The lower values of the equivalent are probably due to the oxidation of the reductone released by hydrolysis.

A number of the condensation products with reductone have been found to be hemihydrated, monohydrated or dihydrated. The reductone-sulphaguanidine condensation product is dihydrated and this is interesting since sulphaguanidine itself is monohydrated. Dehydro-L-ascorbic acid has recently been shown by Kenyon & Munro (1948) to be hydrated. This compound has a structure not unlike the keto form of the condensation products of reductone.

Reactions of reductone condensation products

The reductone-*p*-aminobenzoic acid condensation product reacts readily with phenylhydrazine (O'Meara *et al.* 1947) to give an osazone. This osazone has been prepared and analysed. It possesses a *p*-aminobenzoic acid residue.

Woods (1940) showed that *p*-aminobenzoic acid can inhibit the action of sulphonamide derivatives, its power of inhibition being greatest with sulphanilamide and least with sulphathiazole. In view of these results, we tried the effect of *p*-aminobenzoic acid on the reductone-sulphathiazole condensation product. When these compounds are mixed in hot aqueous sodium acetate which has a pH similar to that found under biological conditions, the sulphathiazole residue is quickly displaced and the reductone-*p*-aminobenzoic acid condensation product results, whereas the reverse process has not been found to take place under similar conditions. A corresponding result was obtained when the reductone-sulphaguanidine condensation product was treated with *p*-aminobenzoic acid. On the other hand, when the reductone-*p*-aminobenzoic acid condensation product is treated with maphenide (marfanil, *p*-aminomethylbenzenesulphonamide) hydrochloride in the presence of excess sodium acetate the *p*-aminobenzoic acid is rapidly replaced by maphenide, the reductone derivative of the latter being formed.

Reductone-p-aminobenzoic acid condensation product, p-2′:3′-dihydroxyprop-2′-enylideneaminobenzoic acid. A solution of reductone (0·4 g.) in water (5 ml.) was shaken with charcoal and filtered. It was then added, with shaking, to a filtered solution of *p*-aminobenzoic acid (0·7 g.) in a mixture of water (10 ml.) and acetone (10 ml.). The clear solution soon became dark red and orange needles were quickly deposited. After 3 hr. the compound was collected, washed with acetone, water and finally acetone, until the filtrate no longer gave a reaction with *p*-dimethylaminobenzaldehyde. The product (0·45 g.) crystallized from dilute acetic acid as orange-yellow needles, m.p. 254°. (Found: C, 53·15; H, 5·2; N, 6·1. Calc. for $C_{10}H_9O_4N.H_2O$: C, 53·3; H, 4·9; N, 6·2%.) It gave absorption maxima in ethanol at 284 mμ. (log ε, 4·21) and 355·5 mμ. (log ε, 2·74). On drying on the water bath, the orange-yellow needles became red with loss of 1 mol. of water.

Titration. The compound (0·057 g.) in 24·9 ml. 0·05N-NaOH was allowed to stand for 15 min. at room temperature, when the solution had become colourless. It was then titrated with 0·05N-H_2SO_4 using phenolphthalein as indicator, when 14·3 ml. of acid were required for neutralization. This gives an equivalent of 108 and mol. wt. of 216, assuming that the reductone is completely enolized. $C_{10}H_9O_4N.H_2O$ requires mol.wt. 225 (compare the titration of reductone by Norrish & Griffiths, 1928); other estimations gave values of 240 and 228. When the compound was left in contact with 0·05N-NaOH for 24 hr. before titration, values of 62, 57 and 57 were found in three estimations.

Colorimetric estimation of purity of the reductone-p-aminobenzoic acid condensation product. Ten ml. of each of a series of aqueous solutions containing 3–15 mg./l. of *p*-aminobenzoic acid were added to 2 ml. portions of a solution of Ehrlich reagent prepared by dissolving *p*-dimethylaminobenzaldehyde (3 g.) in a mixture of conc. H_2SO_4 (7 ml.) and water (100 ml.). The intensity of coloration in each case was measured by means of a Spekker absorptiometer using a blue filter. Concentration of *p*-aminobenzoic acid was plotted against extinction on a graph. Reductone-*p*-aminobenzoic acid condensation product (15 mg.) in 10 ml. 0·1N-NaOH was warmed for a few minutes and then diluted to 1 l.: 10 ml. of this solution were treated with 2 ml. of the Ehrlich reagent and the extinction was again determined. The *p*-aminobenzoic acid content determined from the calibration graph was 58·0%. $C_{10}H_9O_4N.H_2O$ requires 60·9 and $C_{10}H_9O_4N$ 66·1%. Repeat estimations gave values varying from 57 to 58%.

Reductone-sulphathiazole condensation product. 2-(p-2′:3′-Dihydroxyprop-2′-enylideneaminobenzenesulphonamido)thiazole. Sulphathiazole (2·0 g.) in acetone (30 ml.) containing a few drops of 80% acetic acid was added to reductone (0·5 g.) in dilute acetone (30 ml.). After several hours the required *compound* was obtained as yellow needles, which, after washing with water, gave m.p. 232° (decomp.) with some presoftening. (Found: C, 43·0; H, 4·0. $C_{12}H_{11}O_4N_3S_2.\frac{1}{2}H_2O$ requires C, 43·1; H, 3·6%.) This compound lost water when heated at 100° in a vacuum, after which the sulphathiazole residue was estimated by Ehrlich reagent. (Found: sulphathiazole, 80·0. $C_{12}H_{11}O_4N_3S_2$ requires sulphathiazole, 78·5%.)

Reductone-sulphapyridine condensation product. 2-(p′-2′:3′-Dihydroxyprop-2′-enylideneaminobenzenesulphonamido)pyridine. This *compound* was obtained as an orange amorphous powder which did not melt sharply but started to darken and decompose at 194°. (Found: C, 50·6; H, 4·5. $C_{14}H_{13}O_4N_3S.H_2O$ requires C, 49·9; H, 4·4%.)

Reductone-sulphamezathine condensation product. 4:6-Dimethyl - 2 - (p′ - 2′ : 3′ - dihydroxyprop - 2′ - enylideneaminobenzenesulphonamido)pyrimidine. This *compound* was an amorphous green powder, m.p. 200–240° (decomp.). (Found: C, 48·2; H, 4·9; N, 14·8. $C_{15}H_{16}O_4N_4S.H_2O$ requires C, 49·2; H, 4·9; N, 15·3%.)

Reductone-sulphamethylthiazole condensation product. 4-Methyl - 2 - (p′ - 2′ : 3′ - dihydroxyprop - 2′ - enylideneaminobenzenesulphonamido)thiazole. This *compound* consisted of a yellow-orange, amorphous powder which shrivels and decomposes at 166–168°. (Found: C, 45·2; H, 4·3. $C_{13}H_{13}O_4N_3S_2.\frac{1}{2}H_2O$ requires C, 44·8; H, 4·0%.)

Reductone-sulphaguanidine condensation product. p-(2′:3′-*Dihydroxyprop - 2′ - enylideneaminobenzenesulphonyl)guanidine.* This *compound* formed yellow needles, m.p. 152°, which crystallized from water. (Found: C, 37·5; H, 5·1. $C_{10}H_{12}O_4N_4S.2H_2O$ requires C, 37·5; H, 5·0%.)

Reductone-soluseptasine condensation product. Reductone-p-(sodium-α-sulphoethyl)aminobenzenesulphonamide condensa-

*tion product. Reductone-*p*-aminobenzenesulphonamide condensation product.* p-(2′:3′-*Dihydroxyprop-2′-enylideneamino*)-*benzenesulphonamide.* Reductone was found to displace the substituting groups in the N^4 position in the first two compounds. As a consequence all three gave the same condensation product with reductone. The yellow *condensation product* was crystallized from hot water as orange radiating needles which darkened, shrank and finally decomposed at 170–172°. The compounds derived from each of the three sources did not depress the melting point of one another. (In two analyses found: C, 42·0; H, 4·4. C, 40·8; H, 4·6. $C_9H_{10}O_4N_2S.H_2O$ requires C, 41·5; H, 4·6%.)

Reductone-diaminodiphenylsulphone condensation product. Bis- p - (2′ : 3′ - *dihydroxyprop - 2′ - enylideneamino*)*phenylsulphone.* This *compound* consisted of yellow microprisms which started to decompose at 156°, but gave no definite m.p. (Found: C, 50·7; H, 4·8. $C_{18}H_{16}O_6N_2S.2H_2O$ requires C, 50·9; H, 4·7%.)

*Osazone of the reductone-*p*-aminobenzoic acid condensation product.* The solid obtained when a mixture of the condensation product (0·5 g.), phenylhydrazine hydrochloride (1·0 g.) and sodium acetate (1·0 g.) in acetic acid was warmed on the water bath for 0·5 hr. was collected and the *osazone* crystallized from ethanol as yellow needles, m.p. 188–189°. (Found: C, 68·3; H, 5·3; N, 17·0. $C_{22}H_{19}O_2N_5$ requires C, 68·6; H, 4·9; N, 18·1%.) The *p*-aminobenzoic acid content was also estimated by hydrolysis with HCl. Phenylhydrazine was distilled in steam and the residue was treated with Ehrlich reagent and the depth of colour estimated as above. (Found: *p*-aminobenzoic acid, 36·0. $C_{22}H_{19}O_2N_5$ requires 35·6%.)

Action of p-*aminobenzoic acid on reductone-sulphathiazole condensation product.* A mixture of the reductone-sulphathiazole condensation product (0·5 g.), *p*-aminobenzoic acid (0·5 g.), sodium acetate (2 g.) and water (5 ml.) was boiled for a few minutes and filtered whilst hot. On cooling, a yellow precipitate separated and the solution exhibited the green fluorescence characteristic of the reductone-*p*-aminobenzoic acid condensation product. The precipitate was thoroughly washed with hot water to remove *p*-aminobenzoic acid and sulphathiazole. On drying, the yellow solid became orange and gave m.p. 250°, undepressed by authentic reductone-*p*-aminobenzoic acid condensation product.

Reductone-maphenide(marfanil) condensation product. p-(2′:3′-*Dihydroxyprop-2′-enylideneaminomethyl*)*benzenesulphonamide.* A mixture of reductone-*p*-aminobenzoic acid condensation product, maphenide hydrochloride (0·5 g.) sodium acetate (2 g.) and water (5 ml.) was boiled for 2–3 min. with stirring. The hot mixture was filtered and cooled, when a yellow-green solid separated. It crystallized from ethanol as pale green needles, m.p. 183–184°. (Found: C, 46·9; H, 4·8. $C_{10}H_{12}O_4N_2S$ requires C, 46·9; H, 4·7%.) On hydrolysis of this *compound* and diazotization of the product, a solution was obtained which did not couple with β-naphthol. Reductone-*p*-aminobenzoic acid condensation product, gives, by similar treatment, the expected azo compound.

DISCUSSION

The results here recorded provide chemical support for the theory advanced by O'Meara *et al.* (1944, 1947) that the sulphonamides act by uniting with reductone during the active phase of normal bacterial metabolism. According to the view of these authors, *p*-aminobenzoic acid is the normal cell metabolite which unites with reductone and stabilizes it prior to further utilization by the cell. If the place normally taken by *p*-aminobenzoic acid be occupied by a sulphonamide, cellular metabolism ceases.

We have found that under biological conditions of pH, *p*-aminobenzoic acid and all the sulphonamides tested readily form compounds with reductone. These compounds have been isolated and their constitution has been established. It is apparent that the aldehydic group of reductone undergoes condensation with the free primary aromatic amino group of *p*-aminobenzoic acid and the sulphonamides. It is interesting to note that reductone readily condenses with soluseptasine and *p*-(sodium-α-sulphoethyl)-aminobenzene sulphonamide to give, in each case, the reductone-sulphanilamide condensation product. Northey (1940) has expressed the view that 'unless the substituting group in the N^4 position is hydrolysed, reduced or otherwise removed *in vivo* it appears that the derivative will have little if any activity'. The chemical evidence here provided supports this view. Particularly interesting are the interactions of reductone-sulphonamide condensation products with *p*-aminobenzoic acid and of the reductone-*p*-aminobenzoic acid condensation product with maphenide (marfanil). Since, within the biological range of pH, *p*-aminobenzoic acid readily replaces the sulphonamides from their combinations with reductone, a chemical explanation is provided of the inhibition of the bacteriostatic action of the sulphonamides by *p*-aminobenzoic acid demonstrated by Woods (1940). Similarly, the replacement of *p*-aminobenzoic acid, from its combination with reductone, by maphenide, provides a chemical explanation of the facts that *p*-aminobenzoic acid does not antagonize maphenide (Schreuss, 1942; Goldacre, 1944) and that maphenide, unlike the sulphonamides, does not show a time lag in coming into operation (Jensen, Schmith & Brand, 1942). Moreover, these observations enable the mode of action of maphenide to be brought into line with that of the sulphonamides. Maphenide is a relatively strong base, and high concentrations are possible because of its high solubility in water. Both factors will operate so as to facilitate the replacement of *p*-aminobenzoic acid by maphenide in cellular metabolism.

We believe that the reductone-*p*-aminobenzoic acid condensation product system is likely to prove very important in biological synthesis. Reference to biochemical processes, possibly involving reductone and the reductone-*p*-aminobenzoic acid condensation product, has been made elsewhere (Bell, Cocker & O'Meara, 1948), and the synthesis of pteroic acid and its 7-isomer by Forrest & Walker (1948) and of folic acid and its related compounds by Angier *et al.* (1948), lend colour to this view.

SUMMARY

1. Condensation products of reductone have been prepared with *p*-aminobenzoic acid, 2-*p*-aminobenzenesulphonamidothiazole (sulphathiazole), 2-*p*-aminobenzenesulphonamidopyridine (sulphapyridine), 4:6-dimethyl - 2 - (*p*-aminobenzenesulphonamido) pyrimidine (sulphamezathine), *p*-aminomethylbenzenesulphonamide (maphenide, marfanil), 4-methyl-2-(*p*-aminobenzenesulphonamido)thiazole (sulphamethylthiazole), *p*-aminobenzenesulphonylguanidine, 4:4′-diaminodiphenylsulphone, *p*-(disodium-γ-phenyl-αγ-disulphopropyl)aminobenzenesulphonamide (soluseptasine)

$C_6H_5CH(SO_3Na)CH_2CH(SO_3Na)NHC_6H_4SO_2NH_2$,

p-(sodium-α-sulphoethyl)aminobenzenesulphonamide ($Me.CH(SO_3Na)NHC_6H_4SO_2NH_2$) and *p*-aminobenzenesulphonamide. The condensation products of reductone with the three last compounds were identical.

2. It has been shown that the condensation product of reductone and sulphathiazole is decomposed by *p*-aminobenzoic acid. The resultant product is identical with that obtained from reductone and *p*-aminobenzoic acid (*p*-2′:3′-dihydroxy-prop-2′-enylideneaminobenzoic acid), but the reverse process is apparently not possible under similar conditions. Maphenide, however, displaces *p*-aminobenzoic acid from *p*-2′:3′-dihydroxyprop-2′-enylideneaminobenzoic acid.

3. A theory of the mode of action of sulphonamide derivatives is discussed.

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The Micro-estimation of Citric Acid

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The estimation of citric acid by its conversion into pentabromoacetone (Stahre, 1895) has been greatly improved, notably by Pucher, Sherman & Vickery (1936), who introduced a sensitive colorimetric procedure. Recent modifications (Perlman, Lardy & Johnson, 1944; Krebs & Eggleston, 1944; Goldberg & Bernheim, 1944; Hunter & Leloir, 1945; Speck, Moulder & Evans, 1946; Taussky & Shorr, 1947; Natelson, Lugovoy & Pincus, 1947, 1948; Wolcott & Boyer, 1948) have further increased the reliability and sensitivity of the method. However, the fact that modifications of the method continue to appear indicates that satisfaction is not general. Most authors agree that the addition of permanganate is a critical step which must be performed slowly and cautiously.

We became aware of the defects of the method in the course of an investigation of the effect of various mono-, di- and tri-carboxylic acids on the activity of aconitase. This involved the estimation of citric acid in the presence of a large excess of one or more other organic acids. Under these conditions the recovery of citric acid was apt to be low by up to 30%, a result which was but little affected by the various preliminary treatments recommended. It appears from the published data that, although interference with the specificity of the method has been thoroughly investigated, interference with recovery has received less attention.

In the modification here proposed, permanganate is replaced by vanadic acid as oxidizing agent. In our experience this modification has the advantage of greater simplicity, as the oxidizing agent may be added in bulk, of greater elasticity, as the amount of oxidizing agent need not be adapted to concentrations of citric acid varying within a wide range, and of greater freedom from interference by other substrates. In addition, the method is even more specific than the older one; for example, β-hydroxybutyric acid gives no colour.

The use of vanadic acid was suggested by the observation of Pozzi-Escot (1946), who found that the oxidation of citric acid to acetonedicarboxylic acid by chlorate was catalysed by a trace of vanadic acid. The function of chlorate in this reaction is probably that of reoxidizing the reduced vanadyl ion, and it might be expected that in the presence of a sufficient quantity of vanadate the oxidation would proceed in the absence of chlorate. This is indeed the case.

METHOD

Reagents

Sulphuric acid, 27 N-H_2SO_4.

Bromine water, saturated.

Ammonium vanadate, 2% solution, dissolve by warming. The substance tends to crystallize on standing, and before use should be redissolved by warming.

Sodium thiosulphate, 5% solution.

Sodium sulphide (A.R.), 2% solution, freshly prepared.

Sodium sulphate (anhydrous).

Light petroleum, b.p. 80–100°. Purification: extract repeatedly with conc. H_2SO_4, then with a saturated solution of $KMnO_4$ in 0·5 N-H_2SO_4 and finally with a 5% solution of $FeSO_4$ in 0·5 N-H_2SO_4; wash with water, dry and rectify.

Citric acid standard, 1 mg. of the crystalline acid ($C_6H_8O_7.H_2O$)/ml. (in N-H_2SO_4).

Procedure

(*a*) *For quantities of 0·1–1·0 mg. citric acid.* The solution (5 ml., containing not more than about 1 mg. citric acid) is mixed with 27 N-H_2SO_4 (5 ml.) in a 30 ml. test tube. The mixture is cooled to room temperature, and bromine water (2 ml.) and ammonium vanadate solution (3 ml.) are added. The contents are well mixed and the stoppered tube is left in a water bath at 50° for 20 min. A slowly increasing turbidity indicates the formation of pentabromoacetone. The tube is again cooled to room temperature and excess Br_2 is removed by adding 10–12 drops of $Na_2S_2O_3$ solution. The solution, which is now blue or bluish green, is extracted with 6 ml. light petroleum by mechanical shaking of the stoppered tube for 2–3 min. The aqueous layer is blown out with the aid of a wash bottle head, i.e. a rubber stopper fitted with two glass tubes, of which the longer one should be smoothly adjustable so as to reach to the bottom of the test tube. This glass tube is tapered at both ends and the lower tip is ground smooth to ensure an even contact. During its introduction through the petroleum layer the delivery tip is closed with a finger. When most of the aqueous layer has been blown out, the meniscus is allowed to rise slowly in the tube to the delivery tip. Sharp separation is thus achieved. After the addition of a pinch of anhydrous Na_2SO_4 the light petroleum extract is decanted into a clean test tube. Aqueous drops, if any should remain, must be prevented from running out. A 5 ml. portion of the extract is now transferred to a third test tube and shaken for 1 min. with 10 ml. of Na_2S solution. The coloured aqueous layer is blown on to a funnel fitted with a dry filter paper, and the absorption of the clear filtrate is measured in the Spekker absorptiometer or a similar instrument, using 1 cm. cells and Ilford no. 601 (spectrum violet) filters. Na_2S solution is used as a solvent blank. A calibration curve is determined in the usual way.

(*b*) *For quantities of 0·02–0·2 mg. citric acid.* The simplest way of increasing the sensitivity of the method is to reduce the volume of the Na_2S extract. The extent to which this is possible depends mainly on the minimum volume required in the colorimetric instrument available. For quantities of 20–200 μg. citric acid, with a photoelectric test-tube colorimeter (Hilger 'Biochem' absorptiometer), about 3 ml. are needed. The preparation of the pentabromoacetone extract is as described for the 0·1–1·0 mg. range. A 5 ml. sample of the extract is measured into a colorimeter tube and shaken with 3 ml. Na_2S solution. The tube is centrifuged lightly for 1 min. Readings are taken without removing the petroleum layer.

Comments

Precautions. The use of test tubes permits the simultaneous analysis of a large number of samples. The use of a measured portion of the light petroleum extract makes it possible to dispense with repetition of extractions and with washing operations (cf. Natelson *et al.* 1947). Unsatisfactory results can usually be traced to one of two sources of error: incomplete removal of free Br_2 or contamination of the petroleum extract with acid. The former is recognized by the coloration of the petroleum extract; unless this is completely colourless it is better to discard the sample than to complete the reduction of Br_2 by a further addition of thiosulphate. The use of thiosulphate is preferable to that of hydrazine sulphate, as its action is instantaneous and not accompanied by gas evolution which may be troublesome during the subsequent extraction. Contamination with acid may be due to faulty separation or to insufficient cleaning of wash bottle heads or stoppers. It is advisable to keep separate sets of these for the two extractions.

Colour stability. The addition of a stabilizing solvent to the Na_2S extract is unnecessary; likewise no advantage was found in using ice-cold Na_2S solutions, since lower readings were obtained than with solutions at room temperature. For all practical purposes the colour is stable for at least 30 min. at about 18°, especially if protected from strong light. Data on colour fading are contained in Table 1. The fading is slower in the more concentrated solutions.

Table 1. *Colour fading of sodium sulphide extracts after shaking with light petroleum solutions of pentabromoacetone*

Temperature (°)	Exposure to daylight	Time of measurement (hr.) 0	1	2	3	4	5	21	46
		Extinction coefficients							
17–20	+	0·100	0·087	0·081	0·075	0·072	0·071	0·043	—
	−	0·100	0·102	0·099	0·093	0·093	0·086	0·050	—
	+	0·201	0·178	0·155	0·153	0·150	0·147	0·087	0·063
	−	0·201	0·201	0·191	0·188	0·186	0·171	0·103	0·077
	+	1·00	0·99	0·92	0·89	0·87	0·83	0·58	0·390
	−	1·00	1·00	0·96	0·94	0·92	0·90	0·65	0·460
3	−	0·965	0·980	—	—	—	—	—	0·585

The light sensitivity of the colour is a little troublesome when the absorption is measured in tubes with the Hilger 'Biochem' absorptiometer. Here the incident light beam is concentrated on the solution in a sharp focus by a condensing lens. This causes a slow, but completely reversible, decrease of light absorption. Reliable results can be obtained by taking quick readings and by twisting the tube slowly during exposure, thus continuously mixing irradiated and unirradiated parts of the solution. No such colour instability was noticed when flat-sided cuvettes were used.

Accuracy. The calibration curve is a straight line going through the origin. The standard deviation of an estimation is well below 2% within the range of 0·2–1·0 mg. citric acid (Table 2). Standardization of the temperature (18°) and duration (1 min.) of the Na_2S extraction is important for obtaining reproducible results. If the extraction is prolonged for more than 1 min., a progressive reduction of colour intensity occurs.

Table 2. *Analyses of pure citric acid solutions*

(Extraction by 10 ml. Na_2S solution; Ilford filter no. 601.)

Cryst. citric acid (mg.)	No. of estimations	Extinction coefficient (mean)	Standard deviation	Yield of pentabromoacetone (%)
0·10	10	0·1034	0·00414	103
0·20	10	0·2029	0·00314	101
0·30	10	0·3075	0·00425	102
0·50	10	0·5025	0·00403	100
1·00	10	0·9820	0·00350	100

The colour intensity corresponds to a yield of pure pentabromoacetone of slightly over 100%. This is probably connected with the fact that, besides pentabromoacetone, small quantities of other bromoacetones, notably hexabromoacetone, are formed (Goldberg & Bernheim, 1944).

Specificity. The following compounds, in amounts of 0·1 mmol., gave no colour in the test: glucose, fructose, L-tyrosine, L-phenylalanine; and the following acids: glyoxylic, L-lactic, pyruvic, malonic, succinic, fumaric, maleic, tartronic, DL-malic, L-tartaric, α-ketoglutaric, L-α-hydroxyglutaric, crotonic, (±)-β-hydroxybutric, tricarballylic, citraconic, *cis-* and *trans*-aconitic, (±)-*isocitric*, DL-*threo-* and DL-*erythro*-2:3-dihydroxybutyric, L-glutamic, L-aspartic and iodoacetic.

Solutions of acetoacetic acid contaning more than 0·01 mmol./ml. give a yellow colour, but not after a preliminary boiling with acid. A specimen of oxaloacetic acid also gave a positive reaction, 1 ml. of a 0·1M-solution simulating 0·225 mg. citric acid. This reaction was unaffected by preliminary boiling with acid and it may have been due to contamination of the specimen with citric acid or a related substance. Itaconic acid, in contrast to citraconic acid, produces a yellow colour, 1 ml. of a 0·1M-solution being equal in this respect to 0·80 mg. citric acid. This value was not changed by repeated recrystallization; it is, however, much smaller than that reported by Breusch & Tulus (1947).

In the presence of certain aromatic compounds, especially salicylic acid, it is necessary to carry out a preliminary bromination and to remove the bromination product by filtration or by extraction with light petroleum.

Recovery. The following substances, in amounts of 0·1 mmol., did not interfere with the recovery of 1 mg. citric acid by the usual procedure: glucose, pyruvic, succinic, fumaric, maleic, *cis-* and *trans*-aconitic, glutamic and β-hydroxybutyric acids. In the presence of some other substances, notably hydroxy acids, but also fructose, malonic and α-ketoglutaric acids, recoveries were only quantitative if an excess of Br_2 and vanadate was maintained. Undue reduction of Br_2 is recognized by the colour of the solution veering towards green during incubation at 50°. In this case more Br_2 is added in the form of a saturated solution of Br_2 in 9N-H_2SO_4, in portions of 1–2 ml., until the orange colour of the solution, or brown vapours above it, persist. Additional Br_2 (1–7 ml. of the solution) was required in presence of 0·1 mmol. of the following substances: fructose, malonic, lactic, malic, α-hydroxyglutaric, α-ketoglutaric, oxaloacetic, *iso*citric, tartaric, tartronic and 2:3-dihydroxybutyric acids. Provided the addition of bromine was early and sufficient, recoveries of citric acid were quantitative. In these experiments a 3% solution of ammonium vanadate was used.

The presence of any of the substances mentioned in high concentration is a complication which may require some adjustment of the standard procedure as far as the amounts of Br_2 and vanadate solutions are concerned. The vanadic acid method is more adaptable in this respect and gives better results than the permanganate method.

Pre-treatment with periodic acid. Some interfering substances may be removed by a preliminary treatment with periodic acid. These include not only α-glycols, such as tartaric acid, or polyhydroxy compounds, such as mono- or di-saccharides, etc., but also glyoxylic, malonic and tartronic

acids (Sprinson & Chargaff, 1946). The following procedure gave quantitative recovery of citric acid (1 mg.) in the presence of 0·1 mmol. of fructose or tartaric acid: 0·5 ml. of a saturated solution of sodium periodate is added to 3–5 ml. of the neutral solution to be analysed. After standing at room temperature for 15 min. the solution is acidified with 0·3 ml. 10N-H_2SO_4 and a 2M-solution of KI is added dropwise, until the solution is faintly yellow. Iodate formed in the reaction is thereby reduced to I_2. After addition of 0·1 ml. 6% H_2O_2 (to reduce excess periodic acid to I_2) the solution is measured, filtered from separated I_2 and extracted twice with 3 ml. light petroleum. A sample of the filtrate (3–4 ml.) is mixed with 0·2 ml. 30% KBr (to remove excess H_2O_2), made up to 5 ml. and analysed by the usual procedure.

Modifications of the estimation of pentabromoacetone. Some recent modifications of citric acid analysis concern the estimation of pentabromoacetone. This substance is estimated either by its colour reaction with Na_2S or related compounds, or by procedures in which inorganic bromide is liberated and measured by titration or by optical methods. The latter procedures are less specific than the former, as the extract may contain other bromination products which liberate bromide, but do not give the colour reaction with sulphide (cf. Breusch & Tulus, 1947). In the method of Taussky & Shorr (1947) the solution of pentabromoacetone is shaken with ethanolic NaI solution and the liberated I_2 is measured optically. The colour intensity produced, and therefore the sensitivity of the method, is not superior to that of the Na_2S method. The method has further the great disadvantage that the colour intensity slowly increases for several hours (Table 3).

Table 3. *Extinctions of various extracts* (10 *ml.*) *after shaking with light petroleum solutions of pentabromoacetone* (0·897 *mg. in* 5 *ml.*)

Extraction by	Ilford spectrum filter no.	Time of measurement (hr.) 0	1	2	3
		Extinction coefficients			
Na_2S (2%)	601	0·500	0·490	0·475	0·460
Ethanolic NaI (10%)	601	0·445	0·500	0·530	0·540
Thiourea (4%) in borax (2%)	601	0·575	0·420	0·380	0·365
Thiourea (4%) + Na_2S (0·06%)	603	0·540	0·540	0·536	0·533

Natelson *et al.* (1948) have advocated the use of thiourea at pH 9·2, instead of Na_2S. They claim that the colour is stable at room temperature, and that the extinction coefficient is approximately twice that of the Na_2S colour. Using the Spekker absorptiometer with Ilford filter no. 601 (maximum transmission about 440 mμ.) we obtained, with the method of Natelson *et al.*, an extinction coefficient only 10–15% higher than with Na_2S, but the stability of the colour was considerably less (Table 3).

Natelson *et al.* observed a different colour (absorption maximum at 510 mμ.) after prolonged shaking of a neutral thiourea solution with a light petroleum solution of pentabromoacetone. We found that this colour develops rapidly, in about 0·5 min., if the thiourea solution contains 0·06% Na_2S. Under these conditions fading was indeed reduced to a minimum. The extinction coefficient, read with Ilford filter no. 603 (maximum transmission at about 500 mμ.), was, however, substantially the same as that obtained by other methods (Table 3). According to Natelson *et al.* the pink colour (absorption maximum 510 mμ.) of neutral thiourea solutions changes to the yellow colour (absorption maximum 460 mμ.) at alkaline pH and vice versa. However, with a 4% thiourea-2% borax solution containing 0·06% Na_2S the same pink colour is produced as with a neutral thiourea solution.

At present we are not sufficiently impressed by the advantages of the thiourea method to substitute it for the well-tried sulphide method.

Estimation of citric acid in biological fluids

Serum or plasma. The sample (3 ml.) is added slowly, with shaking, to 10% trichloroacetic acid (25 ml.). A portion of the filtrate (20–22 ml.) is transferred to a small conical flask containing one or two glass beads and the neck is lightly plugged with glass wool to prevent splashing. The solution is boiled down to 2–3 ml. on a hot plate and the residue is poured into a test tube and made up to 5 ml. with the rinsings of the flask and glass wool. The further analysis is carried out according to the procedure for the 0·02–0·2 mg. range. Recovery of added citric acid was good (Table 4). A number of analyses on human sera gave results between 15 and 35 μg./ml.

Recently, microcells for use with the Spekker absorptiometer became available to us. They permit estimations on 0·5 ml. solution and proved very useful for the estimation of citric acid in serum. The light petroleum extract was shaken with 1 ml. 2% sodium sulphide solution and about 0·5 ml. of the coloured aqueous layer was withdrawn with a teat pipette and placed in the microcell. When using this modification we had difficulties with trichloroacetic acid as deproteinizing agent. Metaphosphoric acid, too, was unsatisfactory, but tungstic acid filtrates gave consistent results. A disadvantage of tungstic acid filtrates is the appearance of a precipitate of

Table 4. *Recovery of citric acid from horse serum*

Citric acid (μg.) Added	Found	Recovered	Recovery (%)
0	29·2	—	—
20	48·2	19	95
40	69·0	39·8	99·8
60	88·8	59·6	99·3
80	109·0	79·8	99·8

excess tungstic acid in the strongly acid solution in which the oxidation and bromination of citric acid is carried out. This does not interfere with the reaction, but it makes the removal of the aqueous layer by blowing out impossible. Instead, the petroleum layer is sucked off.

Cerebrospinal fluid. The c.s.f. (1 vol.) is mixed with 30% trichloroacetic acid (0·1 vol.), and 1–3 ml. of the filtrate are directly analysed without further pretreatment. Range: 40–80 μg./ml. (cf. Benni, Larsson & Thunberg, 1943).

Urine. Protein, if present, is removed by precipitation with trichloroacetic acid, turbidities by filtration, acetoacetic acid by boiling with dilute acid, and aromatic compounds, such as salicylates, by preliminary bromination. No preliminary treatment whatever is required for normal urines which are analysed directly after suitable dilution. Samples of 1–5 ml. of urine diluted fivefold usually give satisfactory readings. Recovery of added citric acid was quantitative.

SUMMARY

1. A modification of the colorimetric estimation of citric acid is described in which permanganate is replaced by vanadic acid. This gives increased simplicity and speed of operation, and greater freedom from interference by other oxidizable substrates. The yield of pentabromoacetone is almost quantitative.

2. Some recent modifications of the colorimetric estimation of pentabromoacetone are critically examined.

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Studies in Detoxication

27. THE ORIENTATION OF CONJUGATION IN THE METABOLITES OF 4-CHLOROCATECHOL AND 4-CHLORORESORCINOL, WITH SOME OBSERVATIONS ON THE FATE OF (+)-ADRENALINE, PROTOCATECHUIC ACID AND PROTOCATECHUIC ALDEHYDE IN THE RABBIT

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Garton & Williams (1948) have shown that catechol gives rise to monoconjugates, *o*-hydroxyphenylglucuronide and *o*-hydroxyphenylsulphuric acid, in the rabbit. Quinol and resorcinol also form monoconjugates in the rabbit (Garton & Williams, 1949). The object of the present work was to find out which of the two hydroxyls becomes conjugated if a third group X is present in a position *para* to one of the hydroxyls in catechol compounds. We first studied protocatechuic aldehyde (X = CHO), protocatechuic acid (X = COOH) and (+)-adrenaline

(X = CH(OH).CH_2NHMe).

These three compounds formed glucuronides in the rabbit, but we did not succeed in isolating them in a crystalline state. The structure of these glucuronides could not therefore be rigidly proved. With 4-chlorocatechol (X = Cl), however, we succeeded in isolating in high yield a crystalline monoglucuronide whose structure could be determined. The structural

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problem amounted to proving that the isolated glucuronide was either 4-chloro- (I) or 5-chloro-2-hydroxyphenylglucuronide (II):

(I) (II)

Since chlorocatechol formed a monoconjugate, it was interesting also to know whether 4-chlororesorcinol and chloroquinol formed monoconjugates, because the formation of monoconjugates in these compounds raises interesting questions concerning the orientation of conjugation. The study of the isolation and structure of 4-chlororesorcinol glucuronide is also included in this paper. In this case the structural problem involved consisted in proving that 4-chlororesorcinol glucuronide was either 4-chloro- or 6-chloro-3-hydroxyphenylglucuronide. Our studies on chloroquinol will be reported in a future paper.

METHODS AND MATERIALS

Materials. 4-Chlorocatechol hemihydrate (m.p. 81°) was prepared according to Willstätter & Müller (1911), and 4-chlororesorcinol (m.p. 105°) according to Reinhard (1878) (cf. Moore, Day & Suter, 1934). (+)-Adrenaline (m.p. 210°; $[\alpha]_D^{20°}$ +50° in 0·1 N-HCl) was the gift of Burroughs Wellcome and Co. Protocatechuic aldehyde (m.p. 154°) was a commercial sample. Protocatechuic acid (m.p. 197–198°) was prepared in good yield by adding vanillin in small quantities to fused KOH, the method being a slight modification of that of Tiemann & Haarmann (1874).

Animals. The rabbits used were kept on a diet of either 50 g. or 75 g./day of Lever's cubes with water *ad lib.*

Analytical methods. Ethereal sulphates and glucuronic acid in urine were determined as in earlier papers in this series (Williams, 1938; Hanson, Mills & Williams, 1944).

Reference compounds. 4-Chloro-2-methoxyphenyl benzoate (m.p. 78–79°) was prepared according to Jona & Pozzi (1911).

Synthesis of 4-chloro-3-methoxyphenol. This compound was originally described by von Auwers & Pohl (1914–15), who obtained it in small yield by a lengthy procedure. We prepared it by a Sandmeyer reaction from 4-amino-3-methoxyphenol. Resorcinol monomethyl ether (Dey, 1935) was converted through *p*-sulphophenylazo-*m*-methoxyphenol to 4-amino-3-methoxyphenol (Heidelberger & Jacobs, 1919). CuCl obtained from 12·5 g. $CuSO_4.5H_2O$ was dissolved in 20 ml. conc. HCl. This solution was added to the solution of the diazonium compound obtained by treating 3 g. 4-amino-3-methoxyphenol dissolved in 30 ml. 5–6 N-HCl with 1·5 g. $NaNO_2$ at −5°. The mixture was then refluxed for 45 min., whereby N_2 was liberated and a dark-coloured oil separated. This oil was extracted with ether and the extract well washed with water. The phenol was now transferred to 2 N-NaOH by extraction and finally the alkaline extract was acidified with 2 N-HCl and the phenol extracted with ether. After drying with anhydrous Na_2SO_4, treating with charcoal and evaporating the ether, a dark-coloured gum was obtained which slowly crystallized (yield, 1·4 g.). On distillation in a vacuum (140–150°/<1 mm.) 0·8 g. of 4-chloro-3-methoxyphenol was obtained. After recrystallization from acetone-water the phenol was obtained as white plates, m.p. 77–78°. (Found: OMe, 19·6. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%.) Von Auwers & Pohl (1914–15) give m.p. 79–80°.

4-Chloro-3-methoxyphenyl p-toluenesulphonate was obtained in good yield in the usual manner using aqueous NaOH and *p*-toluenesulphonyl chloride. It formed plates, m.p. 65–66°, after recrystallization from aqueous ethanol. (Found: C, 53·9; H, 4·2; Cl, 11·0; S, 10·0. $C_{14}H_{13}O_4SCl$ requires C, 53·8; H, 4·2; Cl, 11·3; S, 10·3%.)

RESULTS

Table 1 shows that both 4-chlorocatechol and 4-chlororesorcinol were excreted completely conjugated with glucuronic and sulphuric acids, the ratio glucuronide/ethereal sulphate being about 6 for chlorocatechol and 3 for chlororesorcinol. With protocatechuic aldehyde and acid, however, there was by no means a complete conjugation, for just over 40% of the aldehyde and about 30% of the acid could be accounted for by conjugation.

In the case of the aldehyde, large amounts of free protocatechuic acid could be isolated from the acidified urine by extraction with ether. This acid no doubt accounts for the rest of the aldehyde. It was also found that two glucuronides were being excreted, one which could be isolated as a gum and appeared to be a protocatechuic acid glucuronide and the other a protocatechuic aldehyde glucuronide which could be isolated as a 2:4-dinitrophenylhydrazone. As these glucuronides were difficult to purify, they were not further studied.

In the case of protocatechuic acid, a considerable amount of this acid can be extracted from the acidified urine with ether. In one experiment we recovered in crystalline form some 30% of the dose. It appears, therefore, that about one-third of the acid fed appears in the urine conjugated and the rest in the free state.

The results with (+)-adrenaline are different from all the others. The ethereal sulphate figures are probably not significant, whereas the glucuronic acid figures suggest that some 20% of the adrenaline fed was excreted as glucuronides. We spent a considerable time studying the nature of these glucuronides, but we did not reach definite conclusions because the glucuronides were non-crystalline. We were, however, able to show that two glucuronides were being excreted, one being a (+)-adrenaline glucuronide giving (+)-adrenaline on hydrolysis, and the other a nitrogen-free glucuronide containing a catechol-like substance, as indicated by absorption spectrophotometry. The second glucuronide no doubt arises from deamination products of adrenaline and it can be separated chromatographically from the first glucuronide. Colour tests indicated that, in the

Table 1. *Glucuronic acid and ethereal sulphate excretion of rabbits receiving 4-substituted catechols orally*

Compound fed	Rabbit no.	Wt. (kg.)	Dose (mg.)	Ethereal sulphate (mg. SO_3/day) Average normal	Ethereal sulphate (mg. SO_3/day) 'Extra'	Glucuronic acid (mg./day) Average normal	Glucuronic acid (mg./day) 'Extra'	Dose (%) excreted as Ethereal sulphate	Dose (%) excreted as Glucuronide
4-Chlorocatechol*	143	2·9	430	35·8	31·2	247	493	14·0	91·0
	145	2·5	370	34·8	41·2	268	407	21·4	87·0
	146	2·15	320	30·0	18·0	217	353	10·5	84·5
4-Chlororesorcinol†	128	3·0	450	18·2	51·1	124	476	20·5	78·8
	129	3·0	450	19·5	71·4	151	459	28·6	76·0
	130	3·0	450	18·3	57·4	182	484	23·0	80·0
Protocatechuic aldehyde†‡	83	3·1	775	18·8	90·7	121	273	20·2	25·1
	114	2·7	636	20·7	50·8	108	242	14·1	27·1
	117	2·4	601	20·2	51·3	123	239	14·7	28·1
Protocatechuic acid†‡	80	3·1	774	25·3	62·1	—	—	15·5	—
	81	2·45	613	24·0	44·2	—	—	13·9	—
	124	3·3	825	29·3	40·1	80	160	9·4	15·4
	125	3·6	900	11·6	76·8	88	219	16·4	19·4
	126	3·6	900	17·3	53·1	102	220	11·4	19·4
(+)-Adrenaline†	68	2·85	710	17·0	13·3	147	142	4·3	18·9
	102	2·9	730	22·6	21·5	148	153	6·7	19·7
	101	2·85	713	33·0	0·0	—	—	0·0	—
	60	2·55	510	25·5	0·0	160	103	0·0	19·1
	67	2·5	500	30·8	15·0	149	157	6·9	29·6
	68	2·9	580	—	—	174	126	—	20·5

* Diet: 75 g. Lever's cubes/day.
† Diet: 50 g. Lever's cubes/day.
‡ The results quoted for these compounds were obtained by Dr G. A. Garton.

gummy (+)-adrenaline glucuronide, the glucuronic acid molecule is attached to one of the phenolic hydroxyl groups of adrenaline.

The metabolites of 4-chlorocatechol

The structure of 4-chlorocatechol glucuronide

The glucuronide gum. Twelve rabbits received collectively 9 g. of chlorocatechol hemihydrate (0·75 g. each) dissolved in water. The 24-hr. urine (1200 ml.) appeared normal in colour and gave an intense Tollens test for glucuronic acid but no reaction with $FeCl_3$ or Benedict reagent. It was made acid to congo red with dilute H_2SO_4 and saturated with $(NH_4)_2SO_4$. To the mixture 50 ml. of 1 : 5 ethanol : ether were added, and the precipitated salts filtered off. The clear filtrate was now exhaustively extracted in a separating funnel with 200 ml. portions of the 1 : 5 ethanol : ether mixture. After drying over anhydrous Na_2SO_4, the yellowish extract was treated with charcoal and filtered. The filtrate was taken to a dark gum (15 g.) *in vacuo* at a low temperature. This gum, which is presumably *4-chlorocatechol glucuronide*, did not crystallize. It formed a crystalline *salt* (m.p. 108–109°) with benzylamine which appeared to contain two molecules of benzylamine. This salt was, however, unstable. 4-Chlorocatechol itself formed a crystalline salt (m.p. 61–62°) with benzylamine. These salts were not further investigated owing to their instability.

The glucuronide gum gave an intense Tollens test but no colour with $FeCl_3$. With 2:6-dichloroquinonechloroimide it gave a deep blue colour at pH 7. The latter test indicates the presence in the compound of a free phenolic hydroxyl group with the position *para* to it unsubstituted.

4-Chloro-2-methoxyphenylglucuronamide. The above gum (12 g.) was dissolved in 100 ml. absolute ethanol and repeatedly methylated with excess ethereal diazomethane for 12 hr. periods at room temperature until the colour test with 2:6-dichloroquinonechloroimide was negative. Filtration and removal of the solvents left a clear gum which did not crystallize. The gum appears to be *4-chloro-2-methoxyphenyl-glucuronide methyl ester*. (Found: OMe, 19·4. $C_{14}H_{17}O_8Cl$ requires OMe, 17·8%.)

This ester (12 g.) was dissolved in the minimum of absolute methanol and the solution saturated with dry NH_3 at 0°. The flask was stoppered and kept at 0° for 12 hr. After that time the whole had set to a gelatinous mass, which crystallized on adding a few drops of acetone and scratching. The crystals (9·2 g.; m.p. 216°) were filtered and washed with 30% ethanol from which solvent it crystallized as long colourless narrow plates, m.p. 218° and $[\alpha]_D^{20°} - 96·15°$ ($c = 0·2$ in 50% aqueous acetone). The *4-chloro-2-methoxyphenyl-β-D-glucuronamide monohydrate* was insoluble in cold water, ether, light petroleum and benzene, slightly soluble in ethanol, soluble in acetone-water mixtures and readily soluble in hot ethanol, water and acetone. (Found: C, 45·0; H, 5·2; N, 3·9; Cl, 10·0; H_2O, 4·6. $C_{13}H_{16}O_7NCl.H_2O$ requires C, 44·4; H, 5·2; N, 4·0; Cl, 10·1; H_2O, 5·1%.) The yield of this amide corresponded to 60% of the chlorocatechol fed.

Hydrolysis of the above amide and isolation of 4-chloroguaiacol. The amide (1·5 g.) was heated on a boiling water bath for 5 hr. with 50 ml. N-HCl. The amide gradually dissolved and eventually a brown oil separated. The mixture was cooled and extracted with ether. After keeping overnight over anhydrous Na_2SO_4, the ethereal extract was treated with charcoal and filtered. The now pale-yellow filtrate was evaporated at room temperature to a partly crystalline solid

consisting of chloroguaiacol. This was benzoylated with benzoyl chloride and NaOH. On pouring the benzoylated mixture into water a solid (0·9 g.) separated. This was recrystallized (charcoal) from ethanol, yielding 0·65 g. of colourless plates, m.p. 72°. Further recrystallization from ethanol raised the m.p. to 78–79° and the compound was identified as 4-chloro-2-methoxyphenyl benzoate. (Found: OMe, 11·9. Calc. for $C_{14}H_{11}O_3Cl$: OMe, 11·8%.) It did not depress the m.p. of authentic 4-chloro-2-methoxyphenyl benzoate (m.p. 78–79°) prepared according to Jona & Pozzi (1911). The other isomer, 5-chloro-2-methoxyphenyl benzoate, melts at 56–58° (Jona & Pozzi, 1911).

The ethereal sulphate of chlorocatechol

The urine of ten rabbits which had received collectively 10 g. of chlorocatechol was collected for 2 days (2·5 l.) and then concentrated to 500 ml. under reduced pressure. The concentrate was saturated with $(NH_4)_2SO_4$ and extracted with 4 × 300 ml. acetone. These extracts, after the addition of 2 g. K_2CO_3 to maintain the solution alkaline, were reduced to 200 ml. *in vacuo*. At this stage the material contained ethereal sulphate and some glucuronide, but gave no test with $FeCl_3$. Much of the glucuronide was thrown out as a gum on pouring the extract into 1 l. dry acetone. The acetone solution was now reduced to 100 ml. and again poured into 1 l. dry acetone. In this way the whole of the glucuronide was removed and the final acetone solution was now reduced to 100 ml. This solution was now diluted with 300 ml. absolute ethanol and then treated with an ethanolic solution of anhydrous oxalic acid. The precipitated urea oxalate was removed, the filtrate made alkaline with ethanolic KOH and then reduced to small bulk, and the whole process repeated. In this way the urea present was removed. Excess oxalic acid was removed by making the concentrate alkaline with ethanolic KOH and filtering off the precipitated potassium oxalate. Finally, a dark brown syrup was obtained which reacted strongly for ethereal sulphates, but gave negative tests for glucuronide and inorganic sulphate. The $FeCl_3$ test was negative, but became intensely positive for catechol on acid hydrolysis. With 2:6-dichloroquinonechloroimide the gum gave an intense blue colour which suggested that the ethereal sulphate was 4-chloro-2-hydroxyphenylsulphuric acid. We were unable to induce the gum to crystallize.

The gum was therefore methylated in the usual way with methyl sulphate and NaOH until the blue colour given by 2:6-dichloroquinonechloroimide had disappeared, and in this way 1·5 g. of a methylated gum containing ethereal sulphate was obtained. The gum was hydrolysed by boiling for 20 min. with 3N-H_2SO_4 and the phenol set free was extracted with ether. The ether yielded 0·8 g. of a reddish gum (Found: OMe, 16·0. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%), which is presumably crude chloromethoxyphenol. This material was benzoylated, but the product would not crystallize. The benzoylated gum was therefore distilled at 80° in a vacuum (<1 mm.) and the straw-coloured distillate crystallized on seeding with authentic 2-methoxy-4-chlorophenyl benzoate. On recrystallization from ethanol 14 mg. of 2-methoxy-4-chlorophenyl benzoate m.p. and mixed m.p. 79° were obtained. (Found: OMe, 11·6. Calc. for $C_{14}H_{11}O_3Cl$: OMe, 11·8%.) This experiment suggests that the ethereal sulphate in chlorocatechol urine is 4-chloro-2-hydroxyphenylsulphuric acid.

THE METABOLITES OF 4-CHLORORESORCINOL

4-chlororesorcinol glucuronide

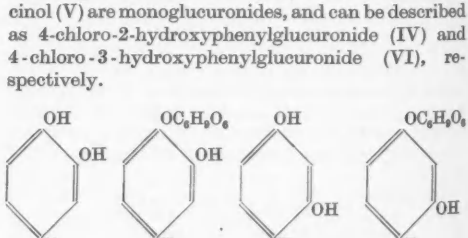
The glucuronide gum. A total of 5·4 g. of 4-chlororesorcinol was fed to six rabbits. The urine (850 ml.) was non-reducing, gave no colour with $FeCl_3$, but gave a strong naphthoresorcinol test. The glucuronide gum (9 g.), presumably *4-chlororesorcinol glucuronide*, was obtained by ether ethanol extraction as described for chlorocatechol glucuronide.

4-Chloro-3-methoxyphenylglucuronamide. Repeated methylation of the gum with ethereal diazomethane yielded the non-crystalline ester, *4-chloro-3-methoxyphenylglucuronic acid methyl ester*. (Found: OMe, 17·9. $C_{16}H_{17}O_8Cl$ requires OMe, 17·8%.) Treatment of the ester in dry methanol with gaseous NH_3 yielded 6·2 g. (equivalent to 50% of the dose) of crude *4-chloro-3-methoxyphenyl-β-D-glucuronamide monohydrate*. The amide formed small plates from water with m.p. 214–215° and $[\alpha]_D^{19°} = -103·3°$ ($c = 0·2$ in 50% aqueous acetone). (Found: C, 44·7; H, 5·3; N, 4·1; OMe, 9·0; H_2O, 4·7. $C_{13}H_{16}O_7NCl.H_2O$ requires C, 44·4; H, 5·2; N, 4·0; OMe, 8·8; H_2O, 5·1%.)

Hydrolysis of the above amide and isolation of 4-chloro-3-methoxyphenol. The above glucuronamide (1·4 g.) was hydrolysed by heating on the water bath for 6 hr. with 50 ml. 2N-HCl. The hydrolysate was black in colour and a black gum separated. It appears that the hydrolysis is complicated by the combination of glucuronic acid and/or its degradation products with the phenol set free, as was found in the case of resorcinol glucuronide (Garton & Williams, 1949). The whole of the hydrolysate was exhaustively extracted with ether. The extracts were washed with water and extracted with 2N-NaOH. The alkaline extract was now acidified with 2N-HCl and the phenol extracted with ether. After drying with anhydrous Na_2SO_4 and treating with charcoal, the ether was evaporated leaving 0·55 g. of a black gum. This gum was now subjected to vacuum distillation (<1 mm.). The fraction distilling at 150–160° (bath temperature) was a pale straw-coloured viscous oil (0·18 g.) which crystallized. On recrystallization from acetone-water, the material was identified as 4-chloro-3-methoxyphenol, m.p. and mixed m.p. 77–78°. (Found: OMe, 19·3. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%.) The *p*-toluenesulphonate was prepared and on recrystallization from aqueous ethanol yielded plates m.p. and mixed m.p. 65–66° with the authentic sample prepared above.

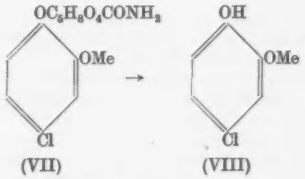
DISCUSSION

It is clear that the glucuronides excreted by rabbits receiving 4-chlorocatechol (III) and 4-chlororesorcinol (V) are monoglucuronides, and can be described as 4-chloro-2-hydroxyphenylglucuronide (IV) and 4-chloro-3-hydroxyphenylglucuronide (VI), respectively.

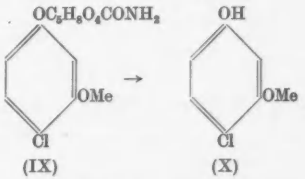


(III) (IV) (V) (VI)

Neither of these compounds was obtained in a crystalline state, but they were readily characterized as the crystalline amides, 4-chloro-2-methoxyphenyl-β-D-glucuronamide (VII; monohydrate, m.p. 218°) and 4-chloro-3-methoxy-β-D-glucuronamide (IX; monohydrate, m.p. 214–215°) which were obtained in excellent yields. The position of conjugation was determined by the hydrolysis of these amides and identification of the resulting chloromethoxyphenols. The amide (VII) yielded 4-chloro-2-methoxyphenol (VIII) characterized as the benzoate, and (IX) yielded 4-chloro-3-methoxyphenol (X) characterized as the *p*-toluenesulphonate.



(VII) (VIII)



(IX) (X)

The present results raise a number of interesting questions concerning the orientation of glucuronic acid conjugation. It has already been shown that in the rabbit catechol, resorcinol and quinol are conjugated on one hydroxyl only (Garton & Williams, 1948, 1949). This has now been shown to apply in the cases of 4-chlorocatechol and 4-chlororesorcinol, but with these compounds a further point arises because the two hydroxyls, in each case, are differently orientated in relation to the third substituent, chlorine. In both cases conjugation appears on the hydroxyl which is *para* to the chloro group. In 4-chlorocatechol the hydroxyl group *meta* to the chloro group is unconjugated, whereas in 4-chlororesorcinol the unconjugated hydroxyl group is *ortho* to the chloro group. It is thus clear that conjugation takes place at the hydroxyl which is farthest from the chloro group. Before, however, we can make definite statements about this orientation we must await results with chloroquinol in which one hydroxyl is *ortho* and the other *meta* to the chloro group. If the orientation of conjugation is simply a matter of distance then it can be predicted that chloroquinol will be conjugated at the *meta* hydroxyl.

It is interesting to compare the conjugation of chlorocatechol and chlororesorcinol with that of catechol and resorcinol. The relevant figures are given in Table 2 which shows that the total conjugation of the chloro compounds is higher than the dihydroxybenzenes. This is clearly the case with the resorcinols, although the ratio glucuronide/ethereal sulphate for chlororesorcinol is approximately the same as for resorcinol. In the case of resorcinol, Garton & Williams (1949) found that at least 10% of the resorcinol fed could be recovered in the urine in the free state. With the two catechols, there appears to be a slight suppression of sulphate conjugation and a slight increase in glucuronic acid conjugation, on going from catechol to 4-chlorocatechol. However, in view of the variations in the individual results it would be safer to conclude that the introduction of the chloro group into the 4-position of catechol has but little influence on the conjugation of catechol, whereas the introduction of the chloro group into the 4-position of resorcinol increases both the ethereal sulphate and the glucuronic acid conjugations.

SUMMARY

1. A study has been made of the conjugation of 4-chlorocatechol and 4-chlororesorcinol in the rabbit.

2. About 87% of 4-chlorocatechol is excreted as a glucuronide, which was characterized as the crystalline 4-chloro-2-methoxyphenylglucuronamide and proved to be 4-chloro-2-hydroxyphenylglucuronide. Some 15% of chlorocatechol forms a mono-ethereal

Table 2. *The conjugation of catechol and resorcinol and their 4-chloro derivatives in the rabbit*

Compound	Dose (mg./kg. body wt.)	Dose (%) excreted as: Ethereal sulphate (*E*)	Glucuronide (*G*)	*G*/*E*	Total conjugation (*G*+*E*)
Catechol*	100†	18	70	3·9	88
4-Chlorocatechol	150†	15·3	87	5·7	103
Resorcinol‡	100†	13·5	52	3·9	65
4-Chlororesorcinol	150†	24	78	3·3	102

* Figures quoted from Garton & Williams (1948).
† In approximately molecular proportions.
‡ Figures quoted from Garton & Williams (1949).

sulphate which is 4-chloro-2-hydroxyphenylsulphuric acid.

3. Similarly, 4-chlororesorcinol forms 78% of 4-chloro-3-hydroxyphenylglucuronide, characterized as 4-chloro-3-methoxyphenylglucuronamide. Some 24% is excreted as ethereal sulphate.

4. The orientation of conjugation in these two compounds is discussed, and it appears that conjugation takes place at the hydroxyl group *para* to the chloro group.

5. (+)-Adrenaline forms 20% of glucuronide but practically no ethereal sulphate. The results suggest that (+)-adrenaline is conjugated on one of its phenolic hydroxyl groups.

6. With protocatechuic acid, some 30% is conjugated and the rest is excreted unchanged.

7. Protocatechuic aldehyde forms two glucuronides, is more highly conjugated than the acid and is largely transferred to protocatechuic acid and its conjugates.

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Occurrence of Cytochrome and Coproporphyrin in Mycobacteria

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Yaoi & Tamiya (1928) and Fujita & Kodama (1934) observed a four-banded spectrum in tubercle bacilli, the bands corresponding closely to those of reduced cytochrome in yeast or heart muscle. Frei, Riedmüller & Almasy (1934) confirmed the description in the case of *Mycobacterium tuberculosis hominis*, but observed a three-banded spectrum lacking component *c* in strains of *Mycobact. avium* and *Mycobact. tuberculosis bovis*. In this laboratory it has been noticed that the absorption bands visible in suspensions of acid-fast saprophytes vary in different species, some of which exhibit a distinct narrow band at 624 mμ. This band could be attributed to the presence of cytochrome a_2; alternatively, it could be due to free porphyrin, the other bands of which are obscured by the cytochrome spectrum.

Fischer & Fink (1925) extracted from heat-killed tubercle bacilli a pigment that was identified spectroscopically as a metal complex of coproporphyrin (bands centred at 522·6 and 560·4 mμ.). The spectrum of free coproporphyrin was not visible. The same authors, working with extracts of crude tuberculin, observed intense absorption bands attributed to protoporphyrin but 'only a very weak coproporphyrin spectrum'. Since the culture media were not known to be free from porphyrin initially, Fischer & Fink (1925) did not regard their results as conclusive evidence of porphyrin formation by tubercle bacilli. The occurrence of porphyrin in acid-fast bacteria was demonstrated unequivocally by Dhéré, Glücksmann & Rapetti (1933), who observed the fluorescence spectrum of coproporphyrin in cells of *Mycobact. smegmatis*, *Mycobact. ranae*, *Mycobact. tuberculosis hominis* and *Mycobact. tuberculosis bovis* (strain B.C.G.).

The work described in this paper, originally undertaken with the object of elucidating the atypical spectra of certain mycobacteria, led to the isolation of coproporphyrin III.

METHODS

Organisms. The following were used: *Mycobact. smegmatis* (no. 523), *Mycobact. phlei* (no. 525), *Mycobact.* sp. *Karlinski* (no. 2071), *Mycobact. ranae* (no. 2891) and *Mycobact. stercoris* (no. 3820). The numbers quoted are the catalogue numbers of the National Collection of Type Cultures, Lister Institute, London. The organisms were cultivated at 38° on meat infusion broth containing 2% (w/v) peptone and 5% (v/v) glycerol. Cultures were harvested on the seventh or eighth day, and washed thrice with distilled water (by centrifuga-

Table 1. *Absorption bands observable in suspensions of mycobacteria*

(Thickness of suspension, 1 cm. Temp., 15–18°. Presence or absence of each band is denoted by + or − respectively.)

	Absorption bands					
Band no.	1	2	3	4	5	6
Centre of band (mμ.)	624	598	570	564	552	526
Organism						
Mycobact. phlei (glycerinated broth)	−	+	−	−	−	−
Mycobact. phlei (non-glycerinated broth)	−	+	−	+	+	+
Mycobact. ranae	−	+	−	+	+	+
Mycobact. Karlinski	+	+	+	+	+	+
Mycobact. smegmatis	+	+	+	+	+	+
Mycobact. stercoris	+	+	+	+	+	+

tion) before use in experiments which required intact organisms. Cultures used for extraction of porphyrin were harvested at the same age by decanting the broth. Bulked cultures were then washed thoroughly with several changes of water, transferred to a linen bag and pressed into a cake.

Spectroscopic examination of bacteria. The moist bacterial mass obtained by centrifuging was packed into an optical cell (internal width, 1 cm.) and illuminated by a 250 W. Philips projection lamp adequately housed. Protection against heat was afforded by a hollow glass cube (side, 3 cm.) filled with water and put between the light source and the optical cell. A small condenser lens was used to concentrate light on the cell. The spectra were observed with a direct vision spectroscope (Schmidt & Haensch) of small dispersion equipped with a wavelength scale. The density of the suspension was adjusted by dilution with water until the definition of the bands was optimal.

Spectrophotometric measurements. Porphyrin solutions were examined in a Beckman quartz spectrophotometer, DU model. The absorption curve of coproporphyrin tetramethyl ester was determined in $CHCl_3$ solution. The ester was hydrolysed by standing in conc. HCl and the absorption curve of the free porphyrin determined in 0·15 N-HCl (see Jope & O'Brien, 1945). The extinction coefficients of the free porphyrin ($E^{1\%}_{1\,cm.}$) were measured at 401 and at 548 mμ., the respective slit widths being 0·04 mm. (≡0·5 mμ. at λ=400 mμ.) and 0·03 mm. (≡0·9 mμ. at λ=550 mμ.).

RESULTS

Absorption spectrum of acid-fast saprophytes

The absorption bands seen in thick suspensions of five saprophytic mycobacteria are listed in Table 1. *Mycobact. Karlinski* and *Mycobact. stercoris* consistently exhibit a sharply defined narrow band (1) at 624 mμ. This band is not present in all cultures of *Mycobact. smegmatis*, and it is absent from the spectra of *Mycobact. phlei* and *Mycobact. ranae*.

Band 2, which is regularly present, extends from 592 to 603 mμ. Stretching from 551 to 568 mμ. there is a zone of absorption which contains two dense bands centred at 552 mμ. (5) and at 564 mμ. (4) respectively. A fainter band (3), centred approximately at 570 mμ., is seen only in *Mycobact. Karlinski* and *Mycobact. stercoris*. Band 6, situated at 520–535 mμ. is partially obscured by end absorption. Bands 2, 4 and 5 were sharply defined in preparations which had been stored at −40°.

When *Mycobact. phlei* is cultivated in glycerol broth it is so deeply pigmented with lipochromes that only one absorption band (2) can be identified; but when the organism is grown on plain broth it is possible to define bands 2, 4, 5 and 6. These results indicate that bands 2, 4 and 5 occupy the positions of the α-bands of cytochromes *a*, *b* and *c*, while band 6 probably includes the β-bands of the *b* and *c* components (Keilin & Hartree, 1939). The intensity of the blue end absorption made it difficult to search for the γ-bands of the cytochromes.

Behaviour of the absorption bands

Effect of aeration. When oxygen was bubbled through suspensions of *Mycobact. Karlinski* and *Mycobact. stercoris*, bands 2, 4, 5 and 6 disappeared, only to reappear after a few minutes of anaerobiosis. Bands 1 and 3 were unaffected, which suggests that they do not belong to a cytochrome complex.

The same phenomena were observed with *Mycobact. smegmatis*, but prolonged aeration was necessary in order to oxidize the cytochromes. Filtered, moist air was passed for 18 hr. through a sterile suspension kept at 37° in a thermostat. Spontaneous reduction at room temperature was slow, but the reduction time was diminished significantly by addition of lactate or malate (Table 2).

Table 2. *Reduction of cytochrome* (Mycobacterium smegmatis)

(Sterile suspension previously aerated for 18 hr. Reduction time assessed by visual observation of intensity of absorption bands.)

Substrate (neutral Na salt, 0·1 M)	Reduction time at 18° (min.)
None	30–40
DL-Lactate	14
L-Malate	15

Oxidation and reduction. The cytochrome bands (2, 4, 5 and 6) disappeared on addition of potassium ferricyanide (two drops of saturated solution/2 ml. bacterial suspension) in all species and reappeared after treatment with a small quantity of solid sodium dithionite ($Na_2S_2O_4$). Bands 1 and 3 (where present) were unaffected by these reagents; nor did these bands change after addition of ammonium sulphide in the absence of ferricyanide (see Fujita & Kodama, 1934).

Effect of oxygen in presence of cyanide. Bands 1 and 3 of *Mycobact. Karlinski* and *Mycobact. smegmatis* were unchanged when oxygen was bubbled through suspensions containing 0·002M-hydrogen cyanide. Bands 2, 4, 5 and 6 persisted in the presence of oxygen and cyanide, behaving like typical cytochrome bands. The experiment shows that band 1 at 624 mμ. is not due to cytochrome a_2 (see Negelein & Gerischer, 1934; Keilin, 1934; Fujita & Kodama, 1934).

Preparation of pyridine haemochromogen. In suspensions of *Mycobact. ranae*, *Mycobact. Karlinski*, *Mycobact. smegmatis* and *Mycobact. stercoris* the cytochrome spectrum was replaced by that of pyridine haemochromogen (dense band at 548–562 mμ. and weaker one at 525 mμ.) following treatment with 2N-potassium hydroxide, a few drops of 10% (w/v) aqueous pyridine solution and solid $Na_2S_2O_4$, added successively. Bands 1 and 3 were unaltered.

Extraction with acetone. When a heavy suspension of *Mycobact. Karlinski* was extracted with acetone, bands 1 and 3 disappeared from the spectrum, although bands 2 and 4 remained visible in moistened acetone powder. After dilution with water the acetone extract was acidified with acetic acid and shaken with ether. The ether layer was faint purplish red, gave a brilliant red fluoresence under a Wood lamp and showed a porphyrin spectrum with bands at 624, 570, 528 and 500 mμ.

These experiments show that the anomalous features of the absorption spectrum encountered in some species of mycobacteria are due to superposition of a porphyrin spectrum upon that of a cytochrome complex containing *a*, *b* and *c* components. Attempts to resolve band 2 into *a* and a_3 components by the use of carbon monoxide (Keilin & Hartree, 1939) were inconclusive.

Isolation of coproporphyrin from Mycobacterium Karlinski

Extraction. Since the band at 624 mμ. was most intense in *Mycobact. Karlinski*, this organism was chosen for an attempt to isolate the porphyrin. Each batch of washed organisms, pressed into a cake weighing about 500 g., was stirred into 500 ml. of cold acetone for 15 min. and filtered on a Büchner funnel. Extraction and filtration were repeated, once with 500 ml. and twice with 250 ml. of acetone, and the several filtrates combined.*

The acetone extract was diluted with 2 volumes of water, acidified with glacial acetic acid (3% of the total volume) and extracted four times with ether. The combined ethereal extracts were shaken with 1·4N-hydrochloric acid, the ether separated, and the porphyrin stored in the acid solution until sufficient had been collected. Prior to storage residual ether was removed *in vacuo* at room temperature. The original acetone extract contained substantial amounts of lipids, which, being ether soluble, were largely removed during the transference of the porphyrin to the acid.

Preliminary purification. In order to remove persistent traces of colourless lipid, the hydrochloric acid solution, representing extraction of 4·8 kg. of bacterial press cake, was neutralized to congo red with solid sodium acetate, acidified with 0·05 volume of glacial acetic acid and the porphyrin transferred to ether. Transference between ether and hydrochloric acid was repeated four times in the usual way until the whole of the porphyrin was concentrated into 150 ml. of 1·4N-hydrochloric acid. Again residual ether was removed *in vacuo* at room temperature.

Sodium hydroxide (30%) was added slowly to bring the solution to pH 3·3 when the porphyrin flocculated. After standing overnight it was separated by centrifugation, dried *in vacuo* over concentrated sulphuric acid, dissolved in 15 ml. of methanol previously saturated with dry hydrogen chloride gas and kept in the ice box for 24 hr. The solution of methyl ester was poured into 150 ml. of chilled water, and the solution extracted repeatedly with small volumes of chloroform. The chloroform solution was washed twice with water, once with 2N-ammonium hydroxide, and then three times with water. After filtration through a paper moistened with chloroform, the solution was evaporated to dryness *in vacuo*.

Chromatographic purification. The crude ester, dissolved in a mixture of benzene (15 ml.) and light petroleum (5 ml.), was adsorbed on a calcium carbonate column (1·5 × 15 cm.) according to the method of Grinstein, Schwartz & Watson (1945). A broad purple band of coproporphyrin methyl

* The completeness of acetone extraction was demonstrated by subsequent treatment of the dry powder from which acetone had been removed *in vacuo*. A sample was extracted with acetic acid and ether, and the ether shaken with a small volume of 2% (w/v) hydrochloric acid. Porphyrin could not be detected in the acid layer either spectroscopically or by fluorescence in ultraviolet light. The success of acetone extraction may depend on the solubility of porphyrin in acetone-soluble lipids of the bacteria.

ester, which migrated slowly down the column, was eluted with the original solvent mixture. The ester was evaporated to dryness *in vacuo* at room temperature, crystallized from anhydrous ether and the crystals dried thoroughly *in vacuo* over concentrated sulphuric acid and paraffin wax.

Since a small amount of pigment remained on the upper and middle zones of the calcium carbonate, the column was cut and both zones extracted with chloroform. The upper zone yielded a quantity of coproporphyrin too small to permit isolation. The middle zone released a brownish red pigment (single absorption band at 645 mμ.) which may be identical with a decomposition product of coproporphyrin described by Rimington (1939). The crystalline coproporphyrin ester was taken up in benzene and chromatography repeated by the method of Gray & Holt (1948). The eluate was evaporated to dryness *in vacuo* at room temperature, and the coproporphyrin ester twice recrystallized from anhydrous ether as before. Yield, 16 mg.

Properties of the crystalline ester. Dark red crystals, assuming the rosette pattern of coproporphyrin III tetramethyl ester (Rimington, 1939). Sharp m.p. at 150°; after cooling, remelt at 172–174°. HCl number, 1·5. Absorption curve (chloroform solution) in the visible region conformed to a 'step-ladder' type with the chief band maxima at (I) 623·5, (II) 567, (III) 534 and (IV) 498·5 mμ. Relative intensities, IV > III > II > I.

Properties of the free porphyrin. Solutions in hydrochloric acid and in ether had the characteristic violet colour of coproporphyrin and gave a strong red fluorescence in ultraviolet light. HCl number, 0·1. Absorption curve in 0·15N-hydrochloric acid showed three bands with maxima at (I) 591, (II) 548 and (III) 401 mμ. Relative intensities, III > II > I. In 0·15N-hydrochloric acid, $E^{1\%}_{1\,cm.} = 7400$ at 401 mμ. and 243 at 548 mμ.

These data show that the crystalline material is a fairly pure specimen of coproporphyrin III tetramethyl ester. The extinction coefficients are about 9% lower than the maximum values quoted by Jope & O'Brien (1945) for recrystallized specimens of coproporphyrin methyl ester, both natural and synthetic. The melting points are within the range recorded for natural specimens.

Coproporphyrin content of mycobacteria

The coproporphyrin content of *Mycobact. Karlinski* was determined by extracting 22 g. of bacterial cake with acetone, transferring the pigment quantitatively through ether to 0·15N-hydrochloric acid and measuring log I_0/I at 548 mμ. Two transfers between ether and 5% (w/v) hydrochloric acid preceded the final concentration in 0·15N-hydrochloric acid. The organism contained 5–8 mg. of coproporphyrin/kg. of bacteria, depending on the batch.

Protoporphyrin was not encountered at any stage during the isolation. If metal complexes or uroporphyrin were present, these, too, escaped detection.

In the course of this work it was found that the acetone powder contains an unstable haem pigment (possibly derived from cytochrome) which is incompletely removed by acetone-hydrochloric acid. This pigment has a single absorption band at 545–555 mμ. and is readily converted into a pyridine haemochromogen.

Other species. The methods employed failed to extract coproporphyrin from *Mycobact. phlei* and *Mycobact. ranae*. Small quantities were obtained from *Mycobact. smegmatis*; *Mycobact. stercoris* contains considerably more than *Mycobact. smegmatis*, though less than *Mycobact. Karlinski*. These findings were consistent with spectroscopic observations on the intact organisms.

Origin of coproporphyrin

Each 400 ml. nutrient broth was extracted with acetic acid-ether and hydrochloric acid before inoculation to ensure that it was free from coproporphyrin. Spectroscopic and fluorescence tests did not reveal the presence of porphyrin. Tests on the broth for haematin by the conversion to pyridine haemochromogen were also negative.

The ability of *Mycobact. Karlinski* to synthesize coproporphyrin was demonstrated by cultivating the organism in a synthetic medium composed of a basal salt mixture (Edson & Hunter, 1943) to which were added lactate (1%), glycerol (0·5%) and sodium citrate (0·001M; final concentrations). The porphyrin bands at 624 and 570 mμ., accompanied by those of the cytochrome spectrum, were seen in the bacteria, and acetone extracted the usual quantity of coproporphyrin.

DISCUSSION

Since the mycobacteria are strictly aerobic, exhibition of the four absorption bands of a typical cytochrome is to be expected. Indeed, Fujita & Kodama (1934) have classified the tubercle organism, along with *Bacillus subtilis* and other aerobic bacteria which possess *a*, *b*, *c* and *d* bands. The observations described in this paper show that *Mycobact. phlei* and *Mycobact. ranae* conform to group I of Fujita & Kodama's classification, whereas *Mycobact. Karlinski*, *Mycobact. smegmatis* and *Mycobact. stercoris* present a composite spectrum containing bands at 624 and 570 mμ. in addition to those of the typical cytochrome components.

A tentative hypothesis that the band at 624 mμ. represented cytochrome a_2 was disproved by experiments, all of which indicate that neither this band nor the band at 570 mμ. can be due to cytochrome components. *A priori* it was unlikely that the

organisms would contain cytochrome a_2, because this pigment has been found only in association with cytochromes a_1 and b_1 (Fujita & Kodama, 1934; Keilin, 1934; Keilin & Harpley, 1941), which are absent from the mycobacteria in question.

Successful extraction of coproporphyrin has explained the origin of the anomalous bands. In consequence of an unusually high intracellular concentration of coproporphyrin two porphyrin bands are visible, although the remainder are probably obscured by the intensity of the *c* and *d* cytochrome bands and by end absorption. The positions of the two visible porphyrin bands indicate that coproporphyrin may be present as neutral salt or as a natural ester, but not as a metal complex.

Coproporphyrin III has been isolated in the form of a tetramethyl ester, the properties of which suggest that it may be contaminated by a small amount of coproporphyrin I. It was suspected that a small quantity of coproporphyrin I (unidentified) had separated on the top of the column during chromatographic purification of the bacterial extract. Although coproporphyrin is known to occur in a variety of bacteria, yeasts and fungi (see Vannotti, 1937), isolation of characterized methyl esters has seldom been reported. Crystalline coproporphyrin esters were obtained by Fischer & Fink (1925) from *Saccharomyces anamensis* and by Kench & Wilkinson (1945), who obtained both natural isomers from brewer's yeast.

The presence of porphyrin in culture filtrates from *Corynebacterium diphtheriae* has attracted attention ever since Coulter & Stone (1931) demonstrated proportionality between toxin production and porphyrin concentration. Pappenheimer (1947) and Pappenheimer & Hendee (1947) have shown that the relationship can depend on the iron content of the medium, and have suggested a connexion between porphyrin and cytochrome *b*, the chief haem pigment observable spectroscopically in suspensions of *C. diphtheriae*. Gray & Holt (1948) have isolated and characterized the porphyrin as coproporphyrin III, which is accompanied by smaller amounts of uroporphyrin I in culture filtrates.

The significance of a substantial production of coproporphyrin by some mycobacteria, and not by others, is not clear. The possibility of a connexion with cytochrome requires exploration. Preliminary experiments with *Mycobact. Karlinski*, grown on synthetic media, indicate that specific organic nutrients are involved in coproporphyrin formation.

SUMMARY

1. The cytochrome spectrum of certain mycobacteria showing *a*, *b* and *c* components is complicated by two absorption bands, one at 624 mμ. and the other at 570 mμ. It has been shown that these bands are due to coproporphyrin.

2. Coproporphyrin III tetramethyl ester (16 mg.) have been isolated from *Mycobacterium Karlinski* (4·8 kg.) by an acetone extraction method. Coproporphyrin is synthesized by the organism.

3. The occurrence of coproporphyrin in other saprophytic mycobacteria has also been studied.

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Quantitative Studies on Mutants of *Bacterium aerogenes* Requiring Nicotinic Acid and Histidine

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In the microbiological assay of amino-acids and vitamins various species of lactobacilli are the micro-organisms most frequently employed. The medium used for the cultivation of the lactobacilli and of most other micro-organisms at present used in assay procedure is complex, requires expensive ingredients and much time in preparation. The mutant strains of fungi such as Neurospora do indeed grow in simple media, but the harvesting of the mycelium is more laborious than either the turbidimetric or acidimetric measurements of bacterial growth. These disadvantages might be overcome by the use of a bacterium, in wild or mutant form, which would grow in simple medium and give a quantitative response to the required growth factor.

The assay of nicotinic acid with a strain of *Proteus vulgaris*, HX 19, was described by Grossowicz & Sherstinsky (1947). The medium contained casein hydrolysate, carbohydrate and mineral salts. The quantitative response of a mutant of *Escherichia coli* requiring arginine to the presence of arginine in simple medium and in the presence of protein hydrolysates has also recently been reported by Lampen & Jones (1947). As a preliminary study of the suitability of such mutants for use in microbiological assay the quantitative response of certain spontaneous and X-ray induced mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*) to nicotinic acid and to histidine has been investigated. The mutants used in the present experiments were prepared and isolated by Devi, Pontecorvo & Higginbottom (1949).

EXPERIMENTAL

Micro-organisms. The mutant strains were all derived from *Bacterium aerogenes*, A 10C, which grew well in a medium containing only glucose and inorganic salts. Of the two strains requiring nicotinic acid, 893 was a spontaneous mutant and 3A3 was obtained by X-irradiation. Both failed to grow in simple medium in the absence of nicotinic acid. The growth requirements for nicotinic acid could be met by the corresponding amide or by relatively large amounts of quinolinic acid.

The three histidine-requiring mutants were obtained by X-irradiation. One, 9B2, would grow, after a lag period, in the simple medium, and has been described as an 'adaptable' mutant (Devi *et al.* 1949). The other two mutants, 297 and 506, were probably identical and failed to grow in the absence of histidine. So far as could be ascertained the growth requirements of these mutants were specific for nicotinic acid or amide or for histidine. Stock cultures of these mutant strains were carried on agar slopes of 'complete' medium.

Media. The 'complete' medium (pH 7·0) contained glucose 10 g.; peptone, 10 g.; yeastrel, 5·0 g. and agar 15 g./l. of tap water.

The 'simple' medium contained glucose 20 g.; $(NH_4)_2SO_4$, 1·0 g.; KH_2PO_4, 3·5 g.; $MgSO_4.7H_2O$, 0·04 g./l. of glass-distilled water. This was later modified to contain glucose, 20 g.; $(NH_4)_2SO_4$, 3·0 g.; KH_2PO_4, 10·5 g.; $MgSO_4.7H_2O$, 0·04 g.; sodium citrate, 18·5 g. in 1 l. glass-distilled water. The pH was maintained at 6·9–7·1 except where stated otherwise.

The basal (simple) medium was prepared in double the above concentration to allow for dilution by the addition of the standard solutions.

Standard solutions. Stock solutions containing 200 μg./ml. were prepared by dissolving 50 mg. L-histidine monohydrochloride, nicotinic acid or nicotinamide in 250 ml. glass-distilled water. The stock solution of quinolinic acid contained 68 mg. of the acid in 250 ml. glass-distilled water and thus contained the mol. equivalent of 200 μg. nicotinic acid/ml. These solutions could be stored in the refrigerator for 2 weeks without deterioration, although the solution of nicotinamide was less stable than that of the corresponding acid. From the stock solutions fresh dilutions were prepared for each experiment.

Inoculum. Nicotinic acid solution (5 ml.) or histidine solution (5 ml.) was added to 5 or 10 ml. basal medium (depending on the micro-organism being used) and the mixture sterilized at a pressure of 10 lb./sq.in. for 10 min. When cool, the solution was inoculated from a stock culture on 'complete' agar medium and incubated for 21–24 hr. at 37°. The culture was then centrifuged and the bacterial sediment washed twice with 10 ml. quantities of sterile quarter-strength Ringer solution. The washed bacteria were suspended in 10 ml. quarter-strength Ringer solution, spun at a low speed to remove clumps, and the uniform suspension suitably diluted so that the turbidity of the inoculum (read on the Spekker absorptiometer) was the same in each experiment. The inoculum was freshly prepared for each assay.

Assay procedure. The assays were carried out in ordinary tubes 6 in. long and $\frac{5}{8}$ in. internal diameter. Graduated doses of the standard solutions in 5 ml. were added to the tubes, 5 ml. of the basal medium (double strength) added to each tube and the contents well mixed. Triplicate tubes were set up for each dose. The tubes were then capped with inverted specimen tubes and sterilized at a pressure of 10 lb./sq.in. for 10 min. When cool 0·05 ml. of the inoculum was added to each tube except the three used as controls.

After shaking well, the tubes were incubated in a water bath at 37°. Turbidimetric measurements of growth using the Spekker photoelectric absorptiometer were made after

incubation for 17 hr. and the results expressed as extinctions (i.e. as the Spekker drum readings). Acidimetric measurements were made after 1, 2 or 3 days incubation, using 0·1, 0·05 or 0·02 N-NaOH with bromothymol blue as indicator. To each tube 1 ml. of 0·04% aqueous solution of bromothymol blue was added and the contents titrated with NaOH containing 100 ml. bromothymol blue/l., using a comparator.

RESULTS

Nicotinic acid-requiring mutants 893 *and* 3 A 3. The response of the strain 893 to doses of 0·02, 0·04, 0·1, 0·2, 0·4 and 1·0 μg. nicotinic acid in 10 ml. basal medium was determined by both acidimetric and turbidimetric methods. The response varied with the method of measurement (Fig. 1). The growth response to the lower doses of nicotinic acid was very poor. This 'lag' varied slightly in different determinations, but usually ceased with doses over 0·2 or 0·3 μg. nicotinic acid. No 'lag' was shown in acidimetric titrations.

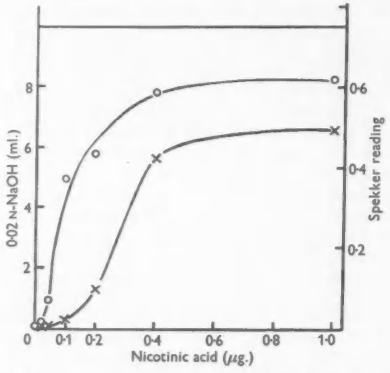

Fig. 1. Dose-response curves, strain 893, to nicotinic acid in the original basal medium; ×—×, turbidimetric measurements after 17 hr. at 37°; O—O, acidimetric measurements after 43 hr. at 37°.

No delay in growth or acid production was obtained with nicotinamide (Fig. 2).

The simplicity of the medium or its low buffering action may have influenced the growth response to nicotinic acid. Therefore, before further study of the mutants, various modifications of the medium, which might increase the extent of growth and acid production while still maintaining a relatively simple composition, were investigated using the parent strain.

Modifications of the basal medium. The extent of growth or acid production was not appreciably affected by increasing the concentration of glucose, by the addition of 0·1% xylose or by increasing the amount of ammonium sulphate present up to 0·6%. A mixture of the purines, adenine, guanine, uracil and xanthine, each in a concentration of 0·001%, inhibited both growth and acid production. Alanine, glycine and glutamic acid (nitrogen equivalent to that in 0·1% ammonium sulphate) did not satisfactorily replace ammonium sulphate as the source of nitrogen, although the addition of asparagine or glutamic acid to the basal medium gave increased growth and acid production.

By the addition of 0·75% vitamin-free casein hydrolysate (Ashe Laboratories Ltd.) to the basal medium, the growth as measured turbimetrically was doubled, although acid production was only increased by one-third. The responses were similar, but much less marked, with a mixture of lysine and glutamic acid in the amounts presumed to be present (Schmidt, 1945) in 0·75% casein hydrolysate. Proline inhibited both growth and acid production, but this inhibition was removed by the addition of lysine.

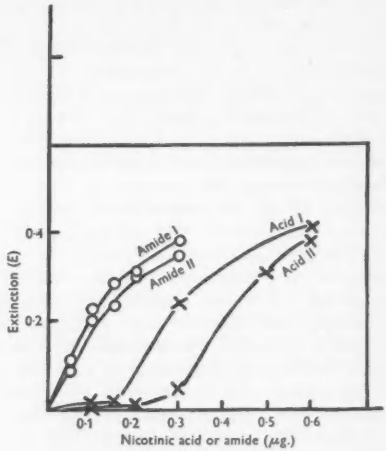

Fig. 2. Dose-response curve, strain 893, to nicotinic acid and amide in original basal medium; turbidimetric measurements (Exps. I and II) after 17 hr. at 37°.

To counter the poorer acid production compared with growth in the presence of casein hydrolysate modification of the buffering agents was investigated. Sodium acetate (0·16–4·0%) or the corresponding amounts of potassium acetate inhibited growth and acid production. The concentration of potassium dihydrogen phosphate was increased by two, three, four and six times that in the original basal medium. The greater the phosphate content the greater the amount of growth and particularly the acid production (titratable acidity). This effect was slightly enhanced by increasing the amount of glucose and ammonium sulphate to 3 and 0·3% respectively (Table 1).

Table 1. *The effect of addition of increased amounts of phosphate and other substances on growth and acid production by* Bact. aerogenes A10C

(Media *A* *B*, *C* and *D* contained respectively two, three, four and six times the amount of KH_2PO_4 in the basal medium.)

Medium	Additions (%)	Turbidimetry after 17 hr. at 37° (Extinction *E*)	Acidimetry after 43 hr. at 37° (ml. 0·05N-NaOH)
A	Nil	0·486	9·5
	$(NH_4)_2SO_4$, 0·2	0·453	9·7
	Glucose, 1	0·463	9·8
	$(NH_4)_2SO_4$, 0·2 + glucose, 1	0·518	10·1
B	Nil	0·570	13·0
	$(NH_4)_2SO_4$, 0·2	0·663	13·7
	Glucose, 1	0·555	14·0
	$(NH_4)_2SO_4$, 0·2 + glucose, 1	0·645	14·3
C	Nil	0·691	15·6
	$(NH_4)_2SO_4$, 0·2	0·720	16·8
	Glucose, 1	0·650	16·5
	$(NH_4)_2SO_4$, 0·2 + glucose, 1	0·661	17·6
D	Nil	0·733	23·3

Although the highest growth response was obtained with a four- to six-fold increase in phosphate, discoloration of the medium occurred during sterilization. To avoid the necessity of sterilizing the glucose and phosphate portions of the medium separately, a threefold increase in phosphate was adopted. Additional buffering associated with a further increase in both growth and acid production was obtained when potassium (2%) or sodium (1·85%) citrate was included in the medium with three times the original amount of phosphate. The reaction in the various modifications of the medium was always adjusted to between pH 6·9 and 7·1.

The final modified basal medium, the composition of which was given on p. 391, contained increased amounts of ammonium sulphate and potassium dihydrogen phosphate and in addition sodium citrate. This basal medium was used for all subsequent quantitative studies of the mutant strains of *Bact. aerogenes*.

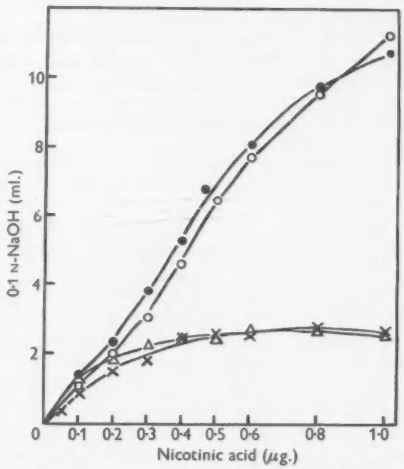

Fig. 3. Dose-response curve, strains 893 and 3A3, with nicotinic acid in original and modified basal medium. Acidimetric measurements after 48 hr. at 37°; ×—×, strain 893 in original medium; ○—○, strain 893 in modified medium; △—△, strain 3A3 in original medium; ●—●, strain 3A3 in modified medium.

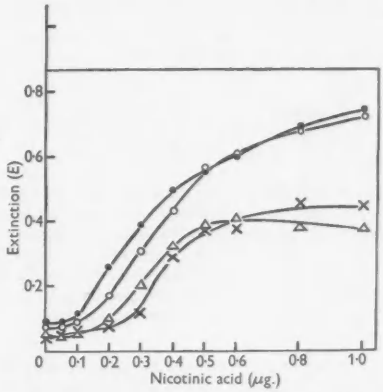

Fig. 4. Dose-response curve, strains 893 and 3A3, to nicotinic acid in original and modified basal medium. Turbidimetric measurements after 17 hr. at 37°; ×—×, strain 893 in original medium; ○—○, strain 893 in modified medium; △—△, strain 3A3 in original medium; ●—●, strain 3A3 in modified medium.

Quantitative response to nicotinic acid and amide. The dose-response curves of strains 893 and 3A3 to nicotinic acid in the original and the modified basal medium are given in Fig. 3 (titratable acidity) and Fig. 4 (turbidity measurement). The maximum

response, whether measured by acidimetric or turbidimetric methods, was greatly improved by the use of the modified medium, but the 'lag' in growth response to small doses of nicotinic acid in the turbidity method remained.

The addition of 0·3% vitamin-free casein hydrolysate to the modified basal medium did not affect the 'lag' in response to nicotinic acid (Fig. 5). Indeed the response was rather less in the presence than in the absence of casein hydrolysate except at the higher dose levels. The growth response to nicotinamide was greater at all dose levels in the presence of casein hydrolysate.

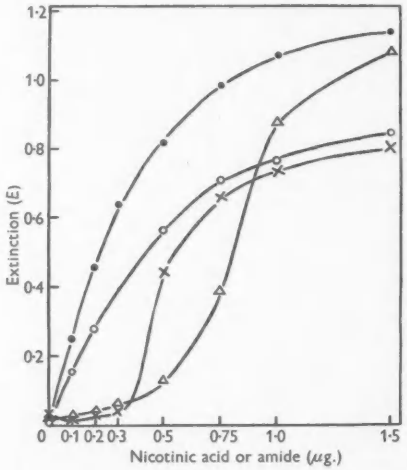

Fig. 5. Dose-response curve, strain 893, with nicotinic acid and nicotinamide in modified basal medium with and without casein hydrolysate. Turbidimetric measurements after 17 hr. at 37°; ×—×, nicotinic acid without casein hydrolysate; ○—○, nicotinic acid with casein hydrolysate; △--△, nicotinamide without casein hydrolysate; ●—●, nicotinamide with casein hydrolysate.

Factors affecting the 'lag' in growth response. In turbidimetric estimations of growth the incubation time was shorter, and Spekker readings were less time-consuming than the acidimetric estimations. Therefore in an attempt to eliminate the 'lag', and thus perfect the turbidimetric measurements of response, the effect of size of inoculum, reaction of the medium and the length of the incubation period at 37° were studied.

Increase in the density of the inoculum tended to reduce the 'lag' somewhat and also increased the growth response to higher doses of nicotinic acid. The reaction of the medium had a definite effect on the response. At pH 6·0 or below the 'lag' was eliminated, but under such conditions the response to higher doses of nicotinic acid was much less than at pH 7·0 (Fig. 6). These results stress the importance of careful control of the reaction of the medium. The 'lag' was markedly reduced or eliminated by increasing the incubation period at 37° from the usual

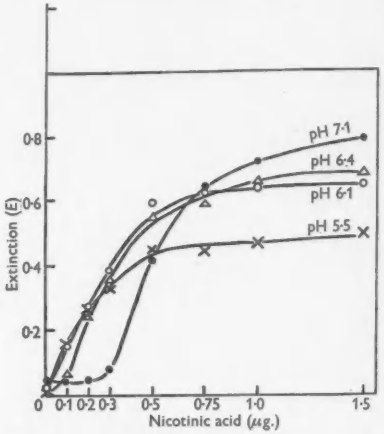

Fig. 6. Effect of hydrogen-ion concentration on the dose-response curve of strain 893 with nicotinic acid; turbidimetric measurements after 17 hr. at 37°.

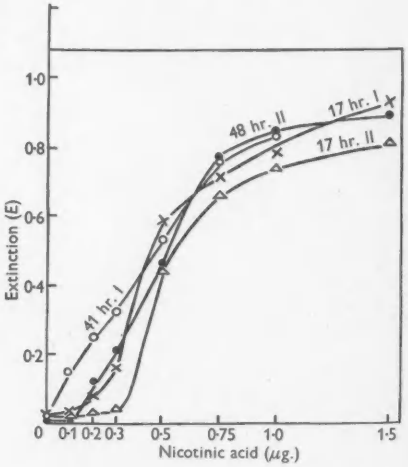

Fig. 7. Effect of period of incubation at 37° on dose-response curve of strain 893 with nicotinic acid; turbidimetric measurements. (Exps. I and II.)

17 hr. to 41 or 48 hr. (Fig. 7). The absence of a 'lag' in the response measured by titration may thus have been due to the use of the longer incubation time of 48 hr. Finally, since there was no 'lag' in response

to the amide, an addition of nicotinamide to give a final concentration of 0·05 μg./assay tube was made to the modified basal medium. The growth response of strains 893 and 3A3 to nicotinic acid showed no 'lag' in the presence of suboptimal amounts of nicotinamide (Fig. 8).

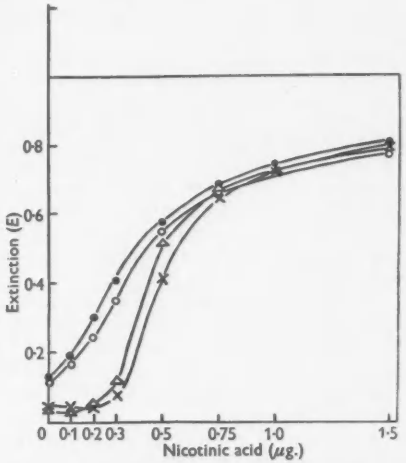

Fig. 8. Dose-response curves, strains 893 and 3A3, with nicotinic acid in modified medium with and without suboptimal amounts of nicotinamide. Turbidimetric measurements after 17 hr. at 37°; ×—×, strain 893 without amide; ○—○, strain 893 with amide; △—△, strain 3A3 without amide; ●—●, strain 3A3 with amide.

Response to quinolinic acid. No growth was obtained with quinolinic acid in doses equivalent to 0·1–1·5 μg. nicotinic acid. With doses of quinolinic acid about 1000 times that of nicotinic acid a response similar to that with nicotinic acid was obtained. It seems likely that this response was due, not to quinolinic acid itself, but to nicotinic acid present as an impurity or formed during the heat sterilization of the quinolinic acid. Similar results were obtained by Koser, Dorfman & Saunders (1940) with strains of *Proteus* sp. and dysentery bacilli which required nicotinic acid.

The histidine-requiring strains 297, 506 *and* 9B2. The response of strains 297 and 506 to 0, 10, 20, 30, 40, 50, 75, 100, 125 and 150 μg. histidine in the modified medium is given in Fig. 9. A smooth curve was given by strains 297 and 506 indicating a quantitative response of both growth and acid production to graded doses of histidine. The response with strain 9B2, an 'adaptable' mutant, was much greater, 10 μg. of histidine giving as much growth as 125 μg. histidine with strains 297 and 506.

The dose-response curves of strain 9B2 with histidine in doses from 0 to 30 μg. are given in Fig. 10. The acidimetric titrations in this instance were recorded after incubation for 19 hr. when about three times as much acid was produced as after incubation for 43 hr. with strains 297 and 506.

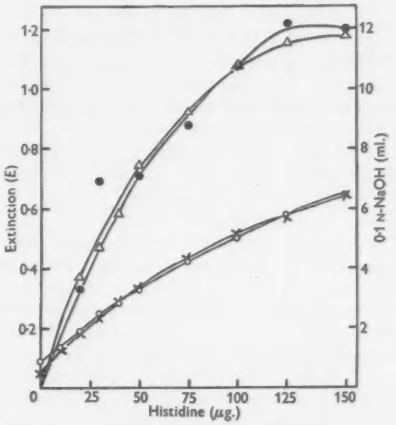

Fig. 9. Dose-response curve, strains 506 and 297, with histidine. Turbidimetric measurements after 17 hr. at 37°; ×—×, strain 506; ○—○, strain 297. Acidimetric measurements after 43 hr. at 37°; △—△, strain 506; ●—●, strain 297.

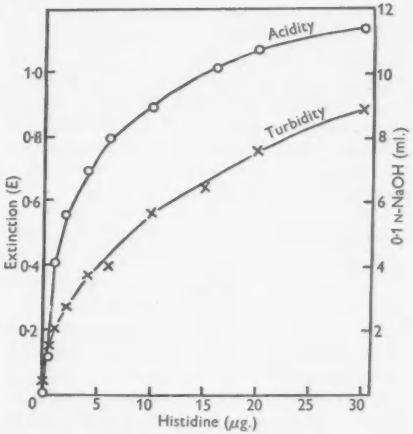

Fig. 10. Dose-response curves of strain 9B2 to histidine; ×—×, turbidimetric measurements after 17 hr. at 37°; ○—○, acidimetric measurements after 19 hr. at 37°.

DISCUSSION

The behaviour of the nicotinic acid-requiring mutants of *Bact. aerogenes*, designated 893 and 3A3, has conformed to that previously described for similar mutants of other micro-organisms. A lack of growth

response, a 'lag', in the presence of low doses of nicotinic acid has been observed by Bonner & Beadle (1946) with nicotinic-requiring mutants of Neurospora. With mutants of *Esch. coli*, Roepke, Libby & Small (1944) found a close correlation between the activity of nicotinic acid at a given pH and the dissociation value of the acid. The dissociation of nicotinic acid decreased with decrease in hydrogen-ion concentration, and it was concluded that only the undissociated acid could enter the cell and be utilized for metabolic purposes.

On the other hand, the relationship between the response to nicotinic acid and the corresponding amide showed a very close parallel with the results obtained in the utilization of glutamic acid and glutamine by *Lactobacillus arabinosus* (*plantarum*) (Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945). As in the present investigation with nicotinic acid and amide, the lag in response to small doses of glutamic acid was not obtained with the corresponding amide. Hac *et al.* (1945) showed that the 'lag' tended to be minimized by increasing the size of the inoculum, by longer periods of incubation and by an acid reaction in the medium. Lyman *et al.* (1945) found that the 'lag' with the acid could be eliminated by the addition of minimal amounts of the amide to the basal medium. They concluded that their results could readily be explained by the assumption that glutamine, rather than glutamic acid, was the substance actually utilized by the micro-organisms. Similarly, it might be concluded that nicotinic acid must be converted to nicotinamide before it can be utilized by the mutants 893 and 3A3. The most important part played by nicotinic acid is in relation to the phosphopyridine nucleotides where it is present as the amide. This would appear to give further support to the suggestion that the first step in the utilization of nicotinic acid is conversion to the amide.

The histidine-requiring strains gave a quantitative growth response to doses of 0–150 μg. of histidine. The 'adaptable' mutant, 9B2, proved very much more sensitive in its response to the histidine. This may be related to its ability to grow in the basal medium in the absence of added histidine after a delay of at least 24 hr.

The application of these mutants to the actual assay of nicotinic acid or amide and of histidine in biological material has not yet been attempted. The non-specific effect of the presence of other substances has not been studied with the mutants requiring histidine. The addition of casein hydrolysate to the basal medium did produce a further increase in growth response to nicotinic acid or amide, and it seems likely that further modification of the basal medium would be required should these mutants be used in microbiological assays.

SUMMARY

1. A study has been made of the quantitative response of certain mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*), which require nicotinic acid and histidine, to the required metabolite in a simple basal medium containing glucose and mineral salts. The effect of making various modifications in the mineral salt content of the medium was investigated.

2. The strains requiring nicotinic acid, after incubation for 17 hr., showed a 'lag' in growth response to doses of nicotinic acid under 0·2–0·3 μg., but not to nicotinamide. The limiting doses at which the 'lag' in growth response ceased varied somewhat in different estimations.

3. The 'lag' was reduced by increasing the size of the inoculum, by lengthening the period of incubation or by increasing the acidity of the medium. It could be eliminated by the addition of minimal amounts of nicotinamide to the basal medium.

4. A quantitative response to histidine in doses from 0 to 150 μg. was obtained with the non-adaptable histidine-requiring mutants. The 'adaptable' mutant showed a much greater response to histidine.

5. The possibility of the use of these mutants in microbiological assay is briefly discussed.

The authors wish to thank Dr E. R. Dawson and his staff at the Research Laboratories of the Distillers Co. Ltd., Great Burgh, Epsom, where facilities were provided for experience in the technique of microbiological assay. The authors also wish to thank Dr G. Pontecorvo of the Department of Genetics in the University of Glasgow, at whose suggestion the study of mutant strains of bacteria was begun and with whose collaboration the mutants used in the present work were isolated. One of us (P. D.) wishes gratefully to acknowledge the tenure of a Scholarship from the Government of India.

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The Composition of Foetal Fluids of Sheep at Different Stages of Gestation

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One of the interesting problems of foetal physiology is the origin, function and interrelation of the allantoic and amniotic fluids. Some information can be obtained by a study of the composition of the two fluids. Most of our knowledge on the subject, in the case of ruminants, is provided by the papers of Jacque (1902), Paton, Watson & Kerr (1907) and Malan, Malan & Curson (1937) on sheep, and of Doderlein (1890) on cows. The older work refers to unspecified material, usually obtained from a slaughterhouse and arranged only according to foetal length or weight. The results of Malan *et al.* (1937) refer to Merino ewes at given stages in gestation and are the most complete. The present work reports the composition of a series of foetal fluids which had been obtained from cross-bred ewes of defined stages in gestation and management during pregnancy. The properties and constituents studied are the specific gravity, dry matter, ash, total and non-protein nitrogen, pH and principal anions and cations of physiological interest.

EXPERIMENTAL

Material. Twelve samples of allantoic and thirteen of amniotic fluid were obtained from some of the ewes in the experiments of Wallace (1948) on the growth of lambs before and after birth. The animals were his 'age series' of ewes. These were of Border-Leicester × Cheviot breed and had been mated with a Suffolk ram. They were fed a standard ration of sanfoin hay and concentrates, which was sufficient to meet requirements for maintenance and reproduction, without fattening the ewe appreciably by the end of pregnancy. They were killed in groups at 28-day intervals during the gestation period.

Treatment of samples. Immediately after slaughter of the ewes, the uterus and contents were removed, the fluids put into beakers and kept in a cold store. Samples for analysis were usually taken within a few days, and stored in waxed tubes with a crystal of thymol at 4° until they could receive attention. The last samples of allantoic fluid contained suspended solid matter, presumably meconium. This was allowed to settle and the supernatant fluid only taken for analysis.

Chemical methods. Specific gravity was determined as the ratio of the weights (to the nearest mg.) of fluid and water in a 10 ml. density bottle at room temperature (17–20°). Dry matter, ash and organic matter were determined by drying 2 ml. of the fluid to constant weight at 110° in a porcelain crucible, ashing as in the Stolte process, described by Peters & Van Slyke (1932), and weighing again.

N was estimated by micro-Kjeldahl, total N on 0·5 ml. allantoic fluid and 1·0 ml. amniotic fluid, and non-protein N on 5·0 ml. filtrate from a mixture of 2·5 ml. fluid and 10 ml. of 10% (w/v) trichloroacetic acid solution; protein N was calculated by difference. In some samples, notably the later ones of amniotic fluid, a clear trichloroacetic acid filtrate could not be obtained, in which case the results are incomplete.

Inorganic ions were estimated by standard methods used in serum analysis as follows: Na (Kramer & Gittleman, 1924); K (Kramer & Tisdall, 1921) precipitation followed by solution in excess ceric sulphate and back titration with 0·02 N-$Na_2S_2O_3$ after adding KI; Ca (Clark & Collip, 1925); Mg (Denis, 1922); chloride (Sendroy, 1937); inorganic P (Fiske & Subbarow, 1925); pH was determined with a quinhydrone electrode and the CO_2-combining capacity in a volumetric Van Slyke apparatus, after equilibrating with alveolar air.

These methods were applied as follows: Ca, Mg, chloride and P were estimated directly on both fluids, and Na directly on the amniotic fluid. Na in the allantoic fluid and K in both fluids were estimated on the ash obtained by the Stolte process (see Peters & Van Slyke, 1932), as direct estimation gave erratic results; when insufficient sample remained to repeat the analysis on the ash, the results are incomplete. Suitable dilutions of the sample were made for the determination of K, Ca, and Mg in the allantoic fluid. In estimating P in the allantoic fluid, difficulty was encountered with samples obtained after the first 2 months of pregnancy, as the values were low and a turbidity developed on the addition of the molybdic sulphuric reagent; however, an approximate value was obtained by extracting the molybdenum blue with *iso*amyl alcohol and comparing with a similarly treated standard.

RESULTS

The results of analyses are given in Table 1, together with details of the samples. The allantoic fluid showed an increase, as gestation proceeded, in specific gravity, non-protein nitrogen and other organic matter, potassium and magnesium, and a decrease in chloride and inorganic phosphorus; the values for calcium were higher in the middle than at the beginning or end of gestation. The amniotic fluid, on the other hand, was much more constant in composition. It had a lower specific gravity, lower organic matter, non-protein and protein nitrogen and magnesium contents, and higher sodium and chloride contents, than the allantoic fluid. The potassium, calcium and phosphorus contents fell within the range of values of this fluid.

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Table 1. *The composition of foetal fluids of sheep at various stages of gestation*

Stage (days)*	No. of foetus*	Wt. of foetus (g.)*	Sex	Quantity of fluid (ml.)*	Sp.gr.	Dry matter	Ash	Organic matter	Total N (g./100 ml.)	N.P.N.	Protein N	pH	Na^+	K^+	Ca^{++}	Mg^{++} (mg./100 ml.)	Cl^-	P (inorganic)	CO_2 combining capacity (ml./100 ml.)
Allantoic fluid																			
28	6	0·47	—	32	1·0082	0·85	0·6	0·25	0·118	0·086	0·032	8·00	—	47	9·7	3·7	230	10·4	102
28	8*a*	0·48	—	18·8	—	0·75	0·6	0·15	—	—	—	8·09	177	21	12·0	2·9	240	13·9	60
56	3*b*	47	—	93	1·0106	1·8	0·45	1·35	0·214	0·185	0·029	7·31	163	20	11·9	6·4	240	4·8	31
56	10	46	—	74	1·0156	2·3	0·6	1·7	0·315	0·210	0·105	6·87	139	38	9·3	8·7	249	7·4	9
84	11*a*	525	M.	74	1·0186	—	—	—	0·350	0·287	0·063	6·89	—	—	24·4	22·9	80	0·9	15
84	11*b*	486	M.	59	1·0174	1·1	0·8	0·3	0·343	0·236	0·107	7·36	—	—	20·7	19·3	113	0·4	22
84	9*b*	490	F.	49	—	—	—	—	0·770	0·546	0·224	6·36	—	—	6·3	19·0	51	<1	—
112	14	1624	F.	308	1·0204	4·3	1·25	3·05	0·323	0·280	0·043	4·49	—	40	46·0	34·1	43	<1	—
112	12*a*	1985	F.	248	1·0218	—	—	—	0·404	0·368	0·036	—	—	—	—	—	—	0·4	—
112	12*b*	2248	M.	422	1·0205	4·15	1·05	3·1	0·374	0·330	0·044	5·14	262	119	9·5	41·0	70	0·5	—
140	7	6226	M.	1050	1·0233	3·9	1·15	2·75	0·360	0·350	0·010	6·46	266	141	4·6	79	42	0·5	15
140	15	5925	F.	1167	1·0202	3·7	1·3	2·4	0·304	0·293	0·011	6·64	86	330	2·5	30·5	40	—	10
Amniotic fluid																			
28	6+2	0·47+0·55	—	0·85+0·87	—	—	—	—	0·08	0·08	0·00	—	—	—	—	—	—	—	—
28	8*a*+*b*	0·48+0·55	—	1·19+1·19	—	—	—	—	—	—	—	—	302	—	—	—	378	—	—
56	3*b*	47	—	164	1·0074	—	—	—	0·038	0·031	0·007	7·47	296	67	7·1	1·4	438	2·8	47
56	3*a*	42	—	184	1·0074	1·15	0·9	0·25	0·038	0·033	0·005	7·47	293	57	6·3	1·3	446	2·7	44
56	10	46	—	149	1·0082	1·0	0·8	0·2	0·061	0·038	0·023	7·39	258	75	6·6	2·2	409	6·1	35
84	11*a*	525	M.	630	1·0074	—	—	—	0·034	0·028	0·006	7·67	309	—	6·9	1·5	424	1·5	45
84	11*b*	486	M.	675	1·0066	1·15	0·8	0·35	0·032	0·029	0·003	7·60	312	37	7·0	1·6	432	1·3	48
84	9*b*	490	F.	602	1·0070	—	—	—	0·035	0·025	0·010	7·12	302	—	6·7	1·4	447	2·0	37
112	14	1624	F.	255	1·0079	1·35	0·85	0·55	0·076	—	—	6·68	287	—	10·8	2·8	448	0·7	—
112	12*a*	1985	F.	738	1·0071	1·5	0·9	0·6	0·055	—	—	4·86	293	53	9·2	3·0	430	3·0	—
112	12*b*	2248	M.	1263	1·0077	1·5	1·15	0·35	0·069	—	—	4·53	284	47	8·2	2·8	416	2·5	—
140	7	6226	M.	1684	1·0078	1·2	0·75	0·45	0·072	—	—	8·40	279	43	5·9	3·4	382	1·3	67
140	15	5925	F.	1416	1·0075	1·1	0·65	0·45	0·073	—	—	6·46	233	33	4·3	2·6	348	1·5	18

* Data quoted from Wallace (1948).

DISCUSSION

Before discussing the results, it is appropriate to consider possible effects of the treatment of the experimental material in terms of conditions and processes obtaining in the womb of the living ewe. Post-mortem changes may set in before the removal of the fluids from the foetal sacs. These might alter the composition through differences in the permeability of the membranes separating the two fluids, or in the functioning of the foetal kidney. The possibility of such changes was emphasized by Jacque (1902), but not explicitly avoided by others. They are considered to be negligible in the present work.

After taking the samples there may be a loss of dissolved gases. Should the fluid contain any appreciable amount of carbon dioxide, the pH value and carbon dioxide content would then both be affected. In the present work, samples could not be obtained anaerobically and so the carbon dioxide combining capacity, rather than the carbon dioxide content, was determined. The pH values reported may consequently be too high. Reliable figures for pH and carbon dioxide content have, therefore, yet to be reported.

During storage of the samples, enzymic changes might occur. Any due to micro-organisms were avoided in the present work. It is not known, however, whether the fluids themselves contain enzymes derived from the foetus. As they are known to contain fructose the possibility of changes, which might affect the acidity, remains. This might have the additional effect in those samples of allantoic fluid, which contained suspended solid matter, of bringing more ions into solution.

The present results are mostly in general agreement with those already reported in the literature, but the following differences may be noted. Malan *et al.* (1937) reported a number of much lower values for the specific gravity, especially of the amniotic fluid. They also found higher values for the phosphorus in the allantoic fluid in the latter part of gestation, but it is not clear whether their results refer to the inorganic, as in the present work, or to the total phosphorus. Doderlein (1890) gives some significantly higher values for the calcium in the amniotic fluid.

The changes in the non-protein nitrogen, organic matter, potassium and calcium contents of the allantoic fluids suggest an increasing excretion of foetal urine as gestation proceeds. This implies that the composition of the allantoic fluid reflects the developing metabolic activity of the foetus, and the developing functional efficiency of its kidney. The decreased chloride in the 84-day samples, and the increased volume of the 115-day samples may therefore indicate the appearance of tubular activity. Although little is known of the composition of ruminant foetal urine, the limited data of Doderlein (1890) and Jacque (1902) support the view that urinary excretion does take place. Jacque (1902), however, reached somewhat different conclusions as to the routes of excretion on the basis of his extensive cryoscopic investigations. He considered that urine was excreted successively via the urachus alone, the urachus and urethra and finally the urethra alone, as gestation proceeded. The present results, however, may not necessarily be incompatible with this. Urinary excretion may not follow a rigid pattern in a given species, and may possibly be affected by variable factors, such as the management of the ewe during pregnancy.

Table 2. *Comparison of average compositions of sheep amniotic fluid and serum*

	Amniotic fluid		Serum*
	(mg./100 ml.)	(m-equiv./l.)	(m-equiv./l.)
Na^+	287	124·8	158·6
K^+	51·5	13·1	8·9
Ca^{++}	7·2	3·6	5·2
Mg^{++}	2·2	1·8	2·8
Total cations	—	143·3	175·5
Cl^-	416·5	117·3	104·2
P (inorganic)	2·3	about 1·0	1·7
CO_2	46·3	20·7	25·0
Total anions	—	139·0	130·9

* Based on data in Shearer & Stewart (1931) and Dukes (1943).

The results for the amniotic fluid justify the calculation of an average composition (cf. Table 2). When expressed as m-equiv./l. the sums of the anions and cations agree to $\pm 1{\cdot}5\,\%$, indicating that the analyses for the main ionic constituents are fairly complete. Inspection of the data suggests comparison with the composition of sheep serum, and an average composition for this fluid, based on the literature, has been included in Table 2. The amniotic fluid is seen to have a slightly lower sodium and higher chloride content, and a calcium content nearer to the ionizable than to the total calcium of serum. This suggests that the amniotic fluid may be a transudate of serum. The possibility may be sufficiently tested for the present, by considering the relations of the predominant ions, sodium and chloride, in the two fluids. The conditions for a Donnan equilibrium are found to hold to $\pm 6\,\%$. To establish this view more definitely, concurrent analyses of amniotic fluids, and maternal and foetal sera from the same uteri, are required. That sodium can readily pass from the maternal serum to the amniotic fluid has been shown for rats and guinea pigs by the radioactive tracer studies of Flexner & Gellhorn (1942). A similar permeability in the structurally different placenta of the sheep would at least allow the possibility of an ionic equilibrium between the two fluids.

A direct relation between the amniotic and allantoic fluids across the placental membranes seems improbable from the present results. Therefore, if the amniotic fluid is absorbed by the foetus, the foetal digestive tract and kidney would be the main agents determining the quantity and composition of the allantoic fluid. Hence the present 'passive' study of the composition of the two fluids gives us the following picture of their origin and interrelation: the amniotic fluid arises as a transudate of the maternal serum and the allantoic fluid comes from the amniotic fluid by the intervention of the foetus. This view could be tested further by a combination of the more active experimental approach of some of the earlier workers with present-day techniques, and would need to be correlated with both physiological and anatomical evidence.

SUMMARY

1. The composition, mainly ionic, of a series of allantoic and amniotic fluids obtained, at five regular intervals during gestation, from ewes fed during pregnancy to maintain their weight to the end of pregnancy, has been studied.

2. As gestation proceeded the allantoic fluid showed an increase in non-protein nitrogen and other organic matter, potassium and magnesium, and decrease in chloride and inorganic phosphorus. The values for calcium were higher in the middle than at the beginning or end of gestation.

3. The amniotic fluid was more constant in composition; it had a lower specific gravity and organic matter, non-protein and protein nitrogen and magnesium contents, and higher sodium and chloride contents than the allantoic fluid. Potassium, calcium and inorganic phosphorus contents were within the ranges for allantoic fluid.

4. Comparison of these results, with data available for the composition of sheep serum and foetal urine, suggest that the amniotic fluid is in Donnan equilibrium with the maternal serum, and that the chemical differences between the amniotic and allantoic fluids are due to the intervention of the digestive tract and kidney of the foetus.

I am indebted to Dr L. R. Wallace, Animal Research Station, University of Cambridge, for the supply of foetal fluids and for permission to include in Table 1 some of his data about the fluids.

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A Photoelectric Flame Photometer

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The flame photometer is an instrument for determining the concentrations of certain metals in solution by measuring the intensity of the light emitted by them when the solution is sprayed under controlled conditions into a non-luminous flame. Lundegårdh (1929–34) developed an apparatus in which cations in solution were sprayed into an air-acetylene flame, and the spectra produced photographed and compared with similar spectra from standard solutions. More recently several workers (for literature, see Barnes, Richardson, Berry & Hood, 1945; Boon, 1945) have designed instruments in which the light from the flame is passed through optical filters on to a photosensitive element (selenium cell or phototube) and the current so produced is measured. Such instruments may be bought in the United States (from the Perkin-Elmer Corporation, Glenbrook, Connecticut, or the National

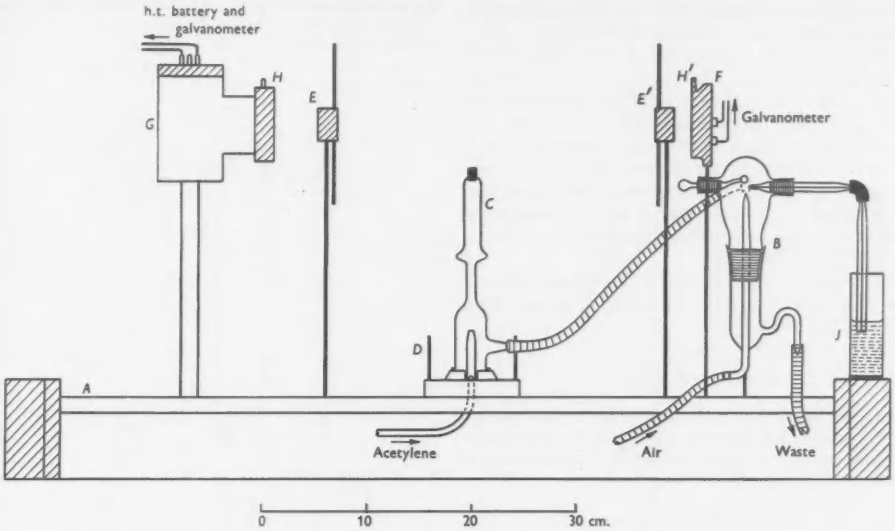

Fig. 1. Flame photometer; general arrangement, excluding chimney, gas cylinders, manometers and galvanometer. *A*, optical bench; *B*, atomizer; *C*, burner; *D*, support for chimney; *E*, *E'*, iris diaphragms; *F*, selenium cell in holder; *G*, phototube in holder; *H*, *H'*, optical filters; *J*, solution under test.

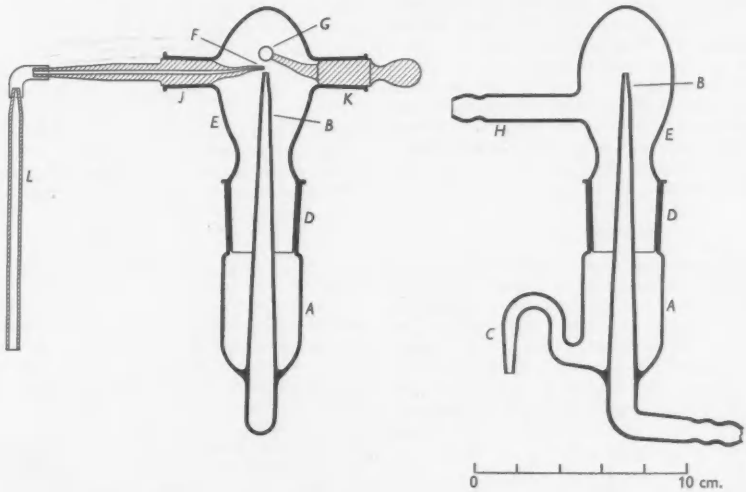

Fig. 2. Atomizer (two vertical cross-sections at right angles). *A*, lower part carrying air inlet (*B*) and drain for waste (*C*). *A* is joined by the ground glass joint (*D*) to the upper part *E*, which carries the solution inlet (*F*), the baffle plate (*G*) and the outlet to the burner (*H*). *F* and *G* are mounted on ground glass joints (*J*, *K*). *F* is joined by a piece of narrow rubber tubing to the vertical tube *L* which dips into the solution to be analysed.

Technical Laboratories, South Pasadena, California), but are difficult to obtain in Europe. The instrument described below can be constructed from parts which are available from stock by any laboratory which has a good mechanic. Its design is based on that of Boon (1945), but, since the latter described his instrument only in a dissertation which is not generally accessible, it was thought desirable to give this account of the construction of our apparatus and its use for the determination of sodium and potassium in biological fluids. The use of the instrument in agricultural chemistry will be described by one of us (W.R.D.) elsewhere.

Preliminary accounts of this work have already been published (Domingo, Klyne & Weedon, 1948; Klyne, 1948, 1949).

CONSTRUCTION

The apparatus consists essentially of an optical bench—carrying an atomizer, a burner, a selenium cell and a phototube with appropriate optical filters (Fig. 1)—gas cylinders, manometers and a galvanometer.

Gas supply and regulation

Air and acetylene are supplied from cylinders carrying two-stage regulators (British Oxygen Co. Ltd.), which regulate the pressures to 10 lb./sq.in. for air and 6 lb./sq.in. for acetylene. Each gas is carried from the cylinder through reinforced rubber tubing (5 mm. internal diameter) to a glass tap (1·5 mm. bore), which is rotated by a slow-motion dial to adjust the gas flow more precisely. (The slow-motion dials are of the type frequently used in radio equipment.) Beyond the glass tap each gas passes to a T-piece, one arm of which is connected to the apparatus whilst the other is connected to a U-tube manometer (height 55 cm.). An air pressure of 40 cm. mercury and an acetylene pressure of 38 cm. water have been found convenient with this apparatus; these pressures are dependent on the size of the air and solution inlets. The glass taps, slow-motion dials and manometers are all conveniently mounted on a vertical board beside the optical bench.

Atomizer

The all-glass atomizer (Fig. 2) resembles that of Rauterberg & Knippenberg (1940), and can be obtained from the Laboratory Glassblowers Co., 63 Lowlands Road, Harrow, Middlesex. Air flowing up the tube *B* past the tip of the solution inlet *F* creates sufficient suction to draw solution up the tube *L* and along *F* and then blow it into a fine spray. The larger particles of this spray strike the plate *G* and are retained; the finer particles pass through *H* and thence to the burner.

Burner

The glass burner (Fig. 3) resembles that of Lundegårdh (1929–34; cf. McClelland & Whalley, 1941; Mitchell, 1948) and can be obtained from the Laboratory Glassblowers Co., Harrow. The dimensions of the atomizer and burner only are critical; the dimensions of the remaining parts of the apparatus can probably be varied somewhat without greatly affecting its performance. The burner is held in position by the metal support (Fig. 4) and is surrounded by a metal chimney (Fig. 5) carrying two holes for a condenser lens and an inspection hole. The centre line of the lens holes is 3·5 cm. above the tip of the burner, and 23·5 cm. above the base of the burner.

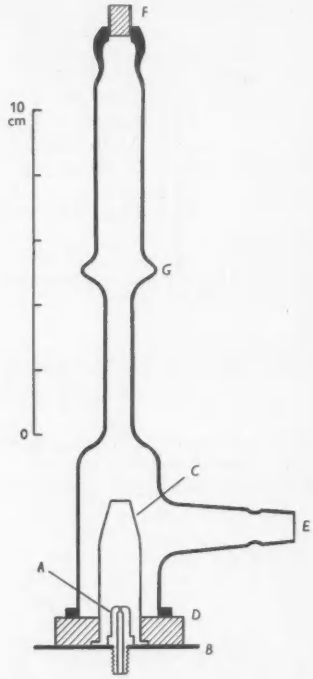

Fig. 3. Burner (vertical section). *A*, acetylene inlet (0·5 mm. diameter) screwed into base plate *B*; *C*, inner cone fitting into rubber washer (*D*); *E*, inlet for air carrying spray from atomizer; *F*, platinum tip; *G*, wider part of tube to fit into support.

Optical and electrical system

The base of the burner and chimney is mounted on an optical bench, one end of which is use for Na determinations and the other for K determinations.

Determination of sodium (Fig. 6). The light from the outer cone of the flame *C* passes through a plano-convex lens *E*, the iris diaphragm *F* and the optical filters *H* on to a selenium cell *J* (type A, 25 mm. diameter, Evans Electroselenium Ltd., Harlow, Essex). The Chance orange filter (no. OY1) with an Ilford blue filter (no. 803) has proved suitable for isolating the Na *D* lines (589 and 590 mμ.). The current from the selenium cell is fed directly on to a galvanometer; a Tinsley SS5 box galvanometer (deflexion 85 mm./μa.) is convenient. We have used a reciprocal logarithmic scale of the type commonly employed on photoelectric colorimeters (cf. King, 1947) graduated in terms of $y = 100 \log (100/x)$.

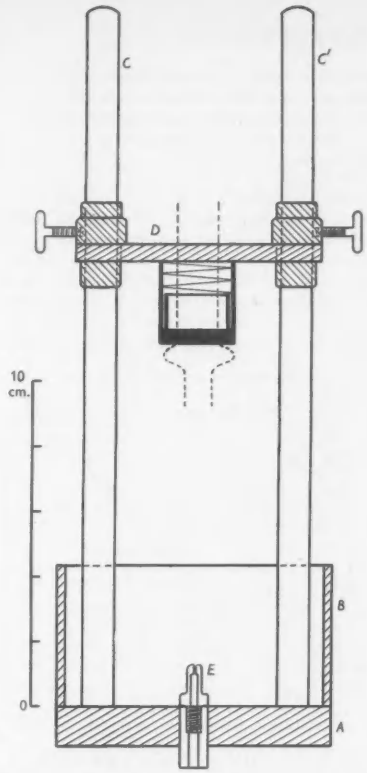

Fig. 4. Support for burner (vertical section). *A*, base plate; *B*, support for chimney with seven air holes (25 mm. diameter); *C*, *C'*, supports; *D*, spring-loaded collar fitting on to wider part of burner (shown in dotted lines); *E*, acetylene inlet.

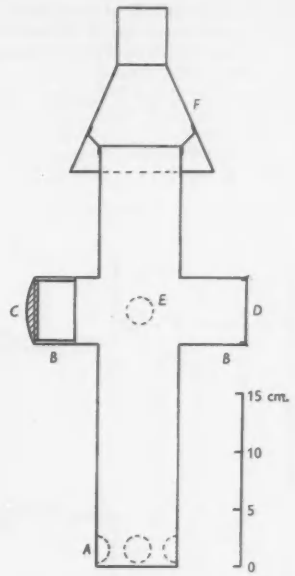

Fig. 5. Chimney (vertical section). *A*, base with holes (each 25 mm. in diameter) to admit air (fits into support Fig. 4 *B*); *B*, *B*, holes for lens; *C*, plano-convex lens (focal length 9 cm.) in sliding tube; *D*, metal plate to cover hole not used for lens; *E*, shows the position of the inspection hole on the front of the chimney; *F*, cowl.

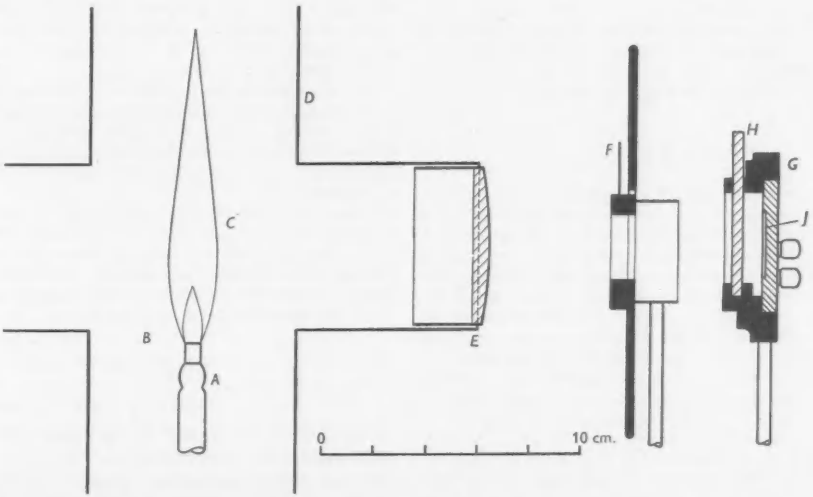

Fig. 6. Optical system for determination of sodium. *A*, burner; *B*, inner cone; *C*, outer cone of flame; *D*, chimney; *E*, plano-convex lens (focal length 9 cm.); *F*, iris diaphragm (maximum aperture, 25 mm.; mounted in shield); *G*, holder for optical filters *H* and selenium cell *J*.

Determination of potassium. The burner and chimney are arranged as for the determination of Na, except that the plano-convex lens is in the left-hand end of the chimney. An iris diaphragm is mounted 15·5 cm. from the centre line of the burner, and a photocell and filter (Fig. 7) are mounted with the centre line of the cell 25 cm. from that of the burner. A gas-filled caesium-cathode cell (Type GS18, Cinema Television Ltd., Crystal Palace, London, S.E.) and an Ilford spectrum deep red filter (no. 609) are suitable for the

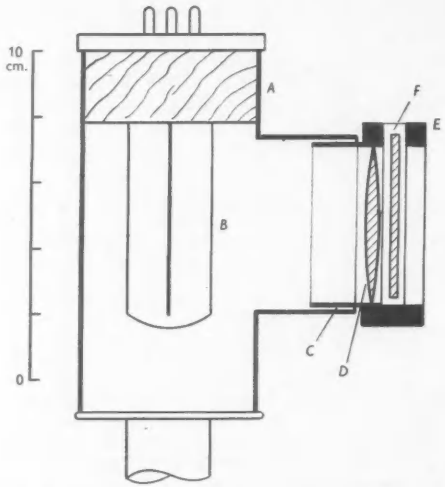

Fig. 7. Photocell and filter for determination of potassium. *A*, holder for photocell *B*; *C*, tube sliding into the front aperture of *A* carrying a biconvex lens *D* (focal length 6 cm.) and a holder *E* which supports the optical filter *F*.

measurement of the 766 and 770 mμ. K lines. A potential of 72 V. is applied to the cell from a dry battery, and the photoelectric current is measured with the same galvanometer as for Na. A resistor and capacitor in parallel (1 MΩ, 0·5 μF., time constant about 1 sec.) are placed in the h.t. lead from the battery to damp variations in h.t. current.

OPERATION

The instrument is assembled without its chimney, and the air is then turned on and adjusted to 40 cm. pressure. Water is placed under the inlet tube and this tube, the air inlet and the baffle plate are adjusted until a maximum spray is produced. The acetylene is then turned on, and the burner is lit. The flame should resemble a non-luminous Bunsen flame and should burn quietly. If flashes of white light appear in the flame, more air is needed. If the flame burns noisily, less air is needed. The behaviour of each burner must be studied individually; careful adjustment of the flame is necessary, since an unsatisfactory flame gives very erratic results.

The chimney is then put in place, and the appropriate photocell connected to the galvanometer. Further adjustment of gas and air pressures may be desirable to steady the flame so as to obtain a steady galvanometer reading.

A standard solution (NaCl or KCl, containing 2–4 mg. Na or K/100 ml. is suitable) is then fed into the atomizer, and the latter is adjusted until the galvanometer gives a maximum reading. The standard solution is replaced by water and the galvanometer is adjusted to read 0. The instrument is now ready for use.

If it were possible to keep the atomizer and burner working in a constant manner, it would be sufficient to calibrate the instrument for each run by passing a series of standard solutions through it (e.g. NaCl solutions containing 0·5, 1·0, 1·5, etc. mg. Na/100 ml.) at the start of the run, and drawing a calibration curve. However, since conditions are liable to vary, we find it best not to rely on a calibration curve; instead, after spraying each sample for analysis, we

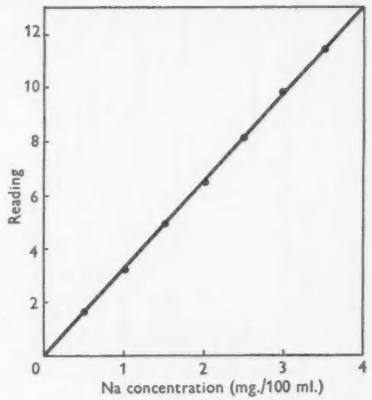

Fig. 8. Flame photometer readings for sodium. Standard curve obtained with pure NaCl solutions. (All readings in Figs. 8–13 are galvanometer readings on the reciprocal logarithmic scale described on p. 402).

spray two standards, one of higher and one of lower concentration, and obtain the result by interpolation. The galvanometer readings on a logarithmic scale are directly proportional to the Na or K concentration, at least over short ranges (Figs. 8 and 13).

The instrument takes in solution at the rate of about 10 ml./min., and a sample with the two appropriate standards can be sprayed twice in 3 min. The instrument responds almost immediately to changes in the solution fed, and it is not necessary to wash through with water between each pair of solutions.

When a series of samples has been sprayed, the instrument is cleaned by thorough spraying with water, and then ethanol is sprayed. While the ethanol is burning at the flame, the acetylene is turned off and then air is drawn through for about a minute. The use of ethanol prevents the flame from back firing when the acetylene is turned off.

RESULTS

Solutions of pure sodium and potassium salts

Over the ranges which are required for sodium and potassium determinations on body fluids (0–5 mg./100 ml.) the relationship between galvano-

meter readings and sodium or potassium concentrations is either linear or so near to linear that simple interpolation between two standards is possible. Specimen results are shown in Figs. 8 and 13.

Interference by anions. In view of the results obtained by other workers on the effect of anions on flame-photometer readings (Barnes *et al.* 1945; Crismon, 1948; Shapiro & Hoagland, 1948) solutions

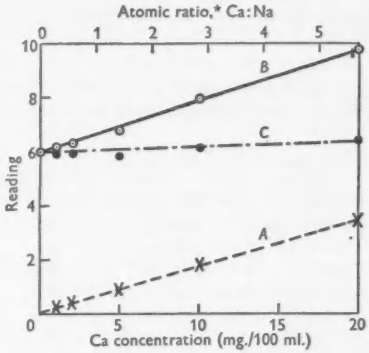

Fig. 9. Flame photometer readings for sodium. Effect of calcium. *A*, readings for calcium (as $CaCl_2$); *B*, readings for calcium solutions, each containing 2 mg. Na/100 ml.; $C = B - A$. (* These values refer to curve *B* only.)

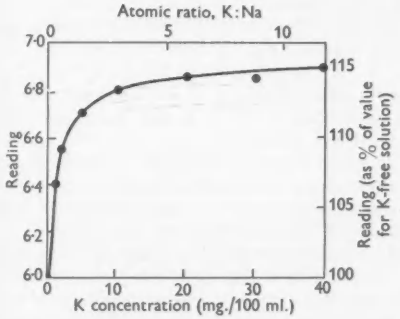

Fig. 10. Flame photometer readings for sodium. Effect of added potassium; 2 mg. Na/100 ml. (as NaCl) in all solutions; K (as KCl) added as shown by abscissae.

of the nitrates, sulphates, dihydrogen phosphates, monohydrogen phosphates, carbonates and acetates of sodium and potassium, each containing 3 mg. Na or K/100 ml. were sprayed. In all cases the galvanometer readings were identical with those for chloride solutions of the same sodium or potassium concentration.

Mutual interference of cations. The following results have been obtained with the instrument described in this paper.

(1) Potassium increases slightly the light emission of sodium (see Fig. 10).

(2) Sodium increases the light emission of potassium (see Figs. 12 and 13). The effect is not due to the sodium *D* line passing the filter, since pure sodium chloride solutions give very small readings (Fig. 12). This effect can be eliminated by the use of a butane-air flame.

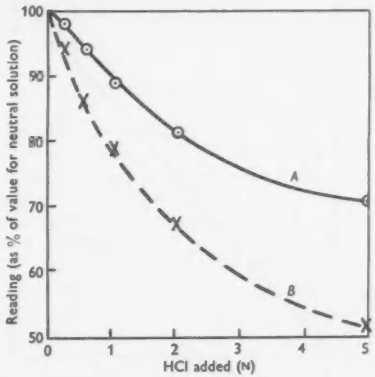

Fig. 11. Flame photometer readings for sodium and potassium. Effect of acid. *A*, 2 mg. Na/100 ml. (as NaCl) in all solutions; *B*, 2 mg. K/100 ml. (as KCl) in all solutions; HCl added as shown by abscissae.

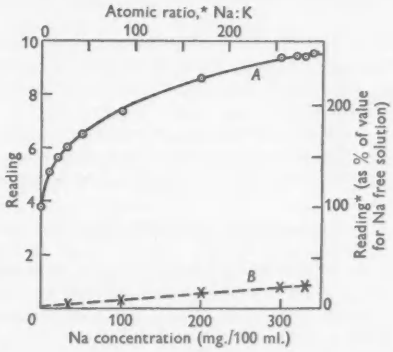

Fig. 12. Flame photometer readings for potassium. Effect of sodium. *A*, solutions containing 2 mg. K/100 ml. (as KCl) + Na (as NaCl) as shown by abscissae; *B*, solutions containing no K, but Na as shown by abscissae. (* These values refer to curve *A* only.)

(3) Some light emitted by calcium ions (presumably the 618 and 620 mμ. lines) passes the sodium filters. The intensity of this light is not sufficient to cause any significant difference in the sodium readings on plasma. Results for solutions containing sodium and calcium show that the

galvanometer readings are the sum of those for the corresponding concentrations of sodium and calcium in separate solutions (see Fig. 9).

(4) No calcium light passes the potassium filter, and there is no interference between calcium and potassium in the flame.

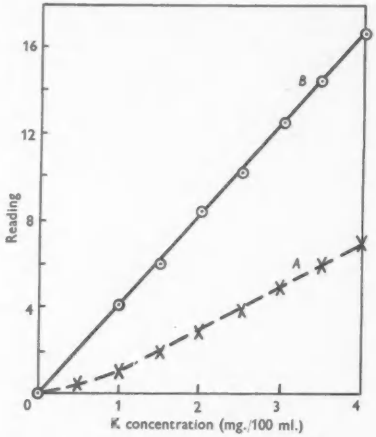

Fig. 13. Flame photometer readings for potassium. Standard curves. *A*, KCl solutions; *B*, KCl solutions each containing 330 mg. Na/100 ml. (as NaCl), used as standards in serum and urine K determinations.

(5) The presence of acid lowers the galvanometer readings for sodium or potassium. This lowering is not significant for acid concentrations below 0·2N (see Fig. 11).

(6) Ammonia added as ammonium chloride (20 mg. NH_3/100 ml.) to solutions had no effect on the photometer readings for sodium or potassium.

Applications in medical biochemistry

The use of the flame photometer in the determination of plasma sodium, serum potassium, urine sodium and urine potassium has been discussed by Hald (1947), who compared the results obtained by the flame photometer and by chemical methods.

Plasma sodium. Plasma (0·2 ml.) is washed from an Ostwald pipette into water (19·8 ml.) giving a 1:100 dilution. These solutions are then sprayed and compared with sodium chloride standards containing 2·5, 3·0 and 3·5 mg. Na/100 ml. It is not necessary to remove protein; and potassium and calcium (in the quantities present in plasma) do not interfere. A specimen protocol showing duplicate readings for test and standard solutions is given in Table 1.

The precision of the method was tested as follows. One hundred samples of standard sodium chloride solutions, of concentrations from 250 to 350 mg. Na/100 ml., representing plasma sodium values, were diluted and sprayed by the method described. The root mean square difference between 'found' and 'calculated' values was 5·4 mg. Na/100 ml. Quality control limits for single observations in routine work are therefore as follows: 90% limits, ±8·9 mg. Na/100ml., 99% limits, ±14·0 mg. Na/100 ml.

Table 1. *Determination of sodium in plasma. Specimen flame photometer readings and results*

Sample	Plasma (diluted 1:100)	Sodium standards (mg. Na/100 ml.) 2·5	3·0	3·5	4·0	Results (mg. Na/100 ml.)	
	Readings	Readings					
A	6·0	—	5·5	6·4	—	328	329
	6·1	—	5·6	6·4	—	330	
B	5·2	4·3	5·4	—	—	291	289
	5·3	4·4	5·6	—	—	287	

Urine sodium. These determinations are carried out as for plasma sodium determinations; 1:100 dilution is usually suitable.

Serum potassium. Normal serum contains about sixteen times as much sodium as potassium, and with this apparatus it is necessary to allow for this by adding sodium to the potassium standards used for comparison. Fig. 12 shows that for a set of sera (*W*, *X*, *Y*, *Z*) containing 20 mg. K/100 ml. and varying concentrations of sodium (say 250, 300, 330 and 350 mg./100 ml.) the flame-photometer readings for potassium after dilution 1:10 with water would show considerable differences. These differences may be reduced to negligible proportions by diluting the sera not with water, but with a solution of sodium chloride containing 330 mg. Na/100 ml. (representing the mean of normal serum sodium values). If the above sera were diluted 1:10 with this solution, the resulting solutions would have the following concentrations (all in mg./100 ml.):

	Original K	Original Na	Final K	Final Na
W	20	250	2·0	322
X	20	300	2·0	327
Y	20	330	2·0	330
Z	20	350	2·0	332

The galvanometer readings for 2·0 mg. K/100 ml. solutions containing 320–340 mg. Na/100 ml. are identical within the limits of experimental error. Thus, if sera are diluted with this solution containing 330 mg. Na/100 ml. and used with potassium standards containing 330 mg. Na/100 ml., the results

are dependent only on the potassium concentrations of the sera.

A series of twenty-two sera were analysed for potassium by this method and by the colorimetric method of Abul-Fadl (1949). The results, with two exceptions, agreed within 2 mg. K/100 ml. and the root mean square deviation was 1·7 mg. K/100 ml. Potassium added to normal serum up to a total concentration of 40 mg. K/100 ml. could be determined with an accuracy of ± 1 mg. K/100 ml.

Urine potassium. If urines are diluted with the 330 mg. Na/100 ml. solution and compared in the flame photometer with the potassium standards used for sera, the results are considerably higher than those obtained by the cobaltinitrite method of Abul-Fadl (1949). However, if the urines are evaporated to dryness and ashed overnight at 400°, flame-photometer determinations on the ashed material show tolerable agreement (within ± 10%) with the cobaltinitrite results (Table 2). Preliminary attempts to trace the cause of the discrepancies in the unashed urine have had no success. The addition of urea (in the quantities commonly found in urine) to potassium standards did not affect the flame photometer results.

Table 2. *Determination of potassium in urine. Comparison of results by flame photometer and colorimetric methods*

(Flame photometer results for unashed urine were obtained by diluting the fresh urine with 330 mg. Na/100 ml. solution. Results for ashed urine were obtained by ashing duplicate samples by the procedure given on this page and carrying out flame photometer and colorimetric determinations (Abul-Fadl, 1949) on the ash.)

	Potassium concentrations (mg./100 ml.)			Differences (%)	
Urine sample	Unashed; flame photometer *a*	Ashed; flame photometer *b*	Ashed; colorimetric *c*	$100\frac{a-c}{c}$	$100\frac{b-c}{c}$
A	92	77	80	+15	−3
		74	70	+37	+6
B	175	153	145	+21	+6
		153	155	+16	−1
C	166	150	155	+7	−3
		144	140	+18	+3
D	237	216	240	+1	−10
		222	245	+3	−9
E	230	197	200	+15	−2
		188	175	+31	+7
F	395	325	300	+31	+8
		380	340	+16	+12

The following method has been found suitable. Urine (2 ml.) is evaporated to dryness in a porcelain crucible on the steam bath. The residue is charred by gentle heating with a Bunsen burner and ashed overnight in a muffle furnace at 400°. The colourless ash is dissolved in 5 ml. of a solution containing 5·43 g. NaCl and 3·65 g. HCl/l. (330mg. Na/100 ml.; 0·1 N in HCl) and the resulting solution (1 or 2 ml., according to K concentration of urine) is diluted to 20 ml. with NaCl solution containing 330 mg. Na/100 ml., giving a final dilution of 1:50 or 1:25 with respect to the original urine. The solutions are then compared with potassium standards containing 330 mg. Na/100 ml.

DISCUSSION

The literature contains a number of references to the mutual interference of metals in flame photometry. The most thorough survey is that of Parks, Johnson & Lykken (1948), who found that in the Perkin-Elmer Model 18 photometer, burning natural gas, sodium readings were lowered by the presence of many metallic salts, including potassium salts, and also by ammonium salts and by acids; potassium readings were lowered by the presence of many metallic salts, including sodium salts, and also by ammonium salts and by acids. Berry, Chappell & Barnes (1946) obtained similar results using an instrument built by the American Cyanamid Co.

Several continental workers (Jansen, Heyes & Richter, 1934; Rauterberg & Knippenberg, 1940; Riehm, 1945, 1948; Boon, 1945) using instruments burning acetylene, which gives a higher flame temperature than the natural gas used in the American instruments, have found, like the present authors, that the foreign metals increase the readings for sodium or potassium. In some cases (Jansen *et al.* 1934; Boon, 1945) the interference effects depended to some extent on the type of optical system used (glass filter, Christiansen filter or monochromator), and some or all of the increase in the readings may have been due to the fact that the optical systems were not isolating the required spectral lines. Riehm (1948) states that sodium increases potassium readings and that potassium increases sodium readings when acetylene is burnt; if, however, illuminating gas is burnt in the same apparatus these two metals do not interfere. The Perkin-Elmer Corporation (1945) also state that with their Model 18 photometer, lithium, sodium and potassium interfere mutually when acetylene is used, but not when illuminating gas is used.

So far as the effects of anions present in the solutions are concerned, Parks *et al.* (1948) and Berry *et al.* (1946) found that the nature of the acids added to solutions of sodium or potassium salts influenced the results considerably. Crismon (1948) found that solutions containing phosphate gave low results for sodium and potassium. Shapiro & Hoagland (1948) found that phosphate had no influence on potassium

results; they pointed out, however, that they were using much lower concentrations than Crismon.

It appears from these scattered and rather confused data that an investigation of these interference effects from the standpoint of the spectroscopist would be desirable.

SUMMARY

1. The construction and operation of a photoelectric flame photometer are described.

2. The use of the photometer for the determination of sodium and potassium in blood and urine is described.

3. The interference between various cations in the flame is discussed with reference to previous work on the subject.

We are indebted to Prof. E. J. King for his interest and encouragement, to Messrs C. Lordan and W. Weedon for constructing the photometer in use at the Postgraduate Medical School, to Messrs R. A. Brennan and J. Gray for much technical assistance, and to Miss S. Tompkins for drawing the figures.

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Vitamin A in Seals

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Specimens of livers of polar bears and bearded seals (*Erignathus barbatus*) collected during the 1939–40 expedition to north-east Greenland (Rodahl, 1943) were found by Rodahl & Moore (1943) to be very rich in vitamin A. During a further expedition to the sealing grounds off Newfoundland and Labrador, carried out under the auspices of the Royal Norwegian Government between March and May 1941, the vitamin A content of seal liver was further investigated. During this expedition the livers of hooded seals (*Cystophora cristata*) and Greenland seals (*Phoca groenlandica*) were weighed and assayed for vitamin A (considerable work on the extraction of vitamin A from this source was also carried out), whilst a limited number of determinations was made on other tissues. The present paper deals also with the results of similar analyses by one of us of material from a single specimen of the Atlantic seal (*Halichoerus grypus*), obtained off the Pembrokeshire coast in October 1946, from two specimens of the common seal (*Phoca vitulina*) obtained in the Wash in August, 1947, and from the four specimens of *Cystophora cristata* collected in the pack-ice east of Greenland in July 1948.

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EXPERIMENTAL

Vitamin A was determined colorimetrically, following the alkaline digestion procedure described by Davies (1933), except for the four 1948 specimens in which the determinations were made spectrographically. All specimens from the sealing grounds off Newfoundland and Labrador were dealt

with in a laboratory on board ship, while those from Pembrokeshire and the Wash were transported to Aberystwyth in large vacuum-walled flasks packed with solid carbon dioxide.

Except in the case of baby seals, the stomachs of all specimens of the four species examined were empty of foodstuffs.

Hooded seals (Cystophora cristata)

A total of sixty livers of this species was examined during March and April 1941. The results are summarized in Table 1.

Table 1. *Livers of hooded seals*

(*a*) Liver weights (g.)

Sex and age	Mean	Range
Baby seals, about 7 days old	1494	1000–2100
Females, fully developed	2929	2200–3750
Males, fully developed	4136	3600–5000

(*b*) Mean liver vitamin A reserves (i.u./g.)

Specimen	No. of animals	March 1941	April 1941	Combined samples
Baby seals				
Foetus	1	120	—	—
1 hr. after birth	1	150	—	—
7 days (approx.)	1	300	—	—
14 days (approx.)	4	1500	—	—
21 days (approx.)	9	—	300	—
Young seals				
1 year (approx.)	2	—	—	2400
2 years (approx.)	2	—	—	1200
Fully developed				
Males above 4 years	26	5180	4320	4934
Females above 4 years	14	1370	2742	2110

Of the total of twenty-six adult male hooded seals, eighteen were examined in March and eight in April, while a total of seven adult females were examined in March, and the same number in April.

RESULTS

There is some evidence to indicate that the vitamin A reserve varies at the different breeding grounds. A further investigation of more material should therefore be carried out at other breeding grounds and at other periods of the year.

In this connexion it may be of interest to note that spectrographical examination of samples of liver from four hooded seals caught in the pack-ice east of Greenland, latitude 74° N., in July 1948, showed lower vitamin A reserves than in the same species caught at the Newfoundland sealing grounds. The results are given in Table 2.

The oil content of the livers of hooded seals up to the age of 3 weeks is usually about 9%. After this age the oil content remains fairly constant at approximately 2·4%. The results of the vitamin A analyses of the liver oil of this species have been combined with the essentially similar results for Greenland seals, and are summarized in Table 6.

Table 2. *Vitamin A content of livers of hooded seals caught in the pack-ice east of Greenland, July* 1948

Sex	Age	Fat in liver (%)	Vitamin A in liver (i.u./g.)
Male	4 months	5·2	450
Female	4 months	3·6	350
Male	16 months	4·3	300
Male	4 years	4·1	450

Greenland seals (Phoca groenlandica)

A total of 145 livers of Greenland seals was examined between March and May 1941. The combined results are shown in Table 3

Table 3. *Livers of Greenland seals*

(*a*) Liver weights (g.)

Sex and age	No. of animals	Mean	Range
Baby seals			
Newborn	1	361	—
8–10 days	1	814	—
Young seals			
Males, 1 year	10	1142	769–1600
Females, 1 year	14	1050	900–1200
Fully developed seals			
Males	17	1783	1600–2250
Females	28	1681	850–2180

(*b*) Mean liver vitamin A contents (i.u./g.)

Sex and age	No. of animals	Mean	Range
Baby seals			
Foetus	1	240	—
8–10 days	7	340	120–600
28 days approx.	10	3000	—
Young seals			
Males, 1 year	24	1620	600–4500
Females, 1 year	23	1560	600–1800
Females, 3 years	10	5000	800–7500
Fully developed seals			
Males above 4 years	47	6570	3000–12000
Females above 4 years	23	2542	600–4500

With some livers separate determinations were made on portions from the right and left quadrate and caudate lobes, but in all instances an even distribution throughout the liver was found.

The possibility of a correlation between liver vitamin A reserve and physiological condition at one time of the year was tested on a series of thirty-six animals caught on 19 and 26 April 1941. In each case a record was made of the approximate age, sex, thickness of subcutaneous fat (blubber) and any abnormalities. In all cases it was noted that the stomach was empty. The results in Table 4 show that no obvious correlation emerged.

Table 4. *Vitamin A content of livers from Greenland seals with reference to physiological conditions*

Sex	Approx. age (years)	Thickness of blubber (cm.)	Liver wt. (kg.)	Oil (%)	Vitamin A content: Liver (i.u./g.)	Vitamin A content: Oil (i.u./g.)	Vitamin A content: Total in whole liver (i.u. × 10^{-6})
			19 April 1941				
Male	>4	2·5	1·9	3·0	750	25,000	1·425
Male	>4	3·5	1·9	2·0	3,000	150,000	5·700
Male	>4	3·3	1·8	2·8	3,000	104,300	5·400
Male	>4	2·3	1·8	2·8	1,800	64,300	3·240
Male	>4	3·5	—	2·4	750	31,250	—
Male	>4	3·4	—	2·0	300	15,000	—
Male	>4	2·3	—	2·0	1,500	75,000	—
Male	>4	3·5	2·1	5·0	12,000	240,000	25·200
Male	4	3·8	2·25	5·0	15,000	300,000	33·750
Male	4	3·5	1·5	4·0	240	6,000	0·360
Male	4	3·5	2·25	3·6	1,200	33,500	2·700
Female	4	4·2	0·85	4·0	2,400	60,000	2·040
Female	4	4·3	1·85	3·0	1,500	50,000	2·775
Female	4	3·8	—	3·0	900	30,000	—
Male	1	1·8	1·25	4·4	900	20,450	1·125
Male	1	3·1	1·25	2·4	1,200	50,000	1·500
Male	1	2·1	1·6	3·0	240	8,000	0·384
Male	1	2·5	—	4·0	750	18,750	—
Female	1	2·5	1·0	2·0	1,500	75,000	1·500
			26 April 1941				
Male	5	3·2	1·75	2·0	1,500	75,000	2·625
Male	5	3·3	1·9	2·0	12,000	600,000	22·800
Male	5	4·3	1·75	3·0	1,500	60,000	2·625
Male	5	4·0	1·7	4·0	2,400	60,000	4·080
Male	5	2·8	1·6	2·0	1,200	60,000	1·920
Female	5	3·5	1·75	3·0	3,000	100,000	5·250
Female	5	3·8	1·65	2·0	750	37,500	1·237
Female	5	4·5	1·5	3·0	1,200	40,000	1·800
Female	5	3·0	2·0	4·0	1,200	38,000	2·400
Female	5	4·1	2·0	4·4	2,400	54,545	4·800
Male	2	3·0	1·25	3·6	300	8,333	0·375
Male	2	2·8	1·35	3·0	1,200	40,000	1·620
Male	3	4·8	1·45	3·6	900	25,000	1·305
Female	1	2·8	1·0	3·6	2,400	66,666	2·400
Female	1	2·3	1·35	3·0	1,500	50,000	2·025
Male	1	4·4	1·1	3·0	3,000	100,000	3·300
Male	1	3·0	1·2	2·0	600	30,000	0·720
			Mean results				
Male	5	3·2	1·82	2·7	3,208	151,588	7·975
Male	4	3·3	2·0	4·2	5,480	113,166	12·147
Male	1	2·4	1·09	3·4	1,165	39,023	1·487
Female	5	3·8	1·78	3·3	1,710	52,309	3·098
Female	4	4·1	1·35	3·3	1,600	55,000	2·408
Female	1	3·3	1·1	2·3	1,700	68,333	1·840

The fat contents of the livers showed a distribution in relation to age essentially similar to that of hooded seals, and are summarized in Table 5.

Table 5. *Age and sex in relation to the fat content of the liver of Greenland seals*

Sex	Age	Mean fat (%)	Range (%)
Female	4 years	3·2	3·0–4·5
Male	4 years	2·8	2·0–5·0
Male	2 years	3·6	—
Male	1 year	3·4	2·4–4·4
Female	1 year	2·3	2·0–3·0
Female	8–10 days	10·0	—

The results of vitamin A analyses on the liver fat of both species are combined in Table 6 which indicates that there was in most instances an inverse relationship between the amount of oil and its vitamin A potency.

The distribution of vitamin A in the organs of a single specimen of Greenland seal was studied, with the results shown in Table 7. The vitamin A reserve in the liver of this individual was only moderate and below the mean value for comparable specimens. Thus only small amounts were detected in the retina, kidney, lung and subcutaneous fat. The pancreas gave a positive reaction, but the con-

centration of vitamin A was too low to permit an accurate determination. The figures are in agreement with unpublished findings on organs of bearded seals (*Erignathus barbatus*) collected during the 1939–40 expedition to north-east Greenland.

Table 6. *Fat content of Greenland seal and hooded seal livers in relation to vitamin A potency*

Group no.	No. of animals	Oil (g./100 g.)	Mean vitamin A content: Liver (i.u./g.)	Mean vitamin A content: Oil (i.u./g.)
1	20	2·0–2·4	3,675	134,687
2	3	2·5–2·9	2,000	104,300
3	10	3·0–3·4	1,459	51,444
4	4	3·5–3·9	1,200	53,374
5	7	4·0–4·4	1,470	38,500
6	8	5·0–5·4	13,500	270,000
7	8	9·0–9·4	300	33,300

Table 7. *Distribution of vitamin A in organs of a normal Greenland seal killed 4 May* 1941

Organ	i.u./g.
Liver	2400
Kidney	6
Lung	3
Spleen	0
Pancreas	Trace
Testes	0
Muscle	0
Cerebellum	0
Cerebrum	0
Heart	0
Thyroid	0
Epididymis	0
Subcutaneous fat	12
Lymph gland	0
Corpus vitreum	0
Retina	4

The vitamin A content of a sample of milk collected from a female with a baby in its second week of life was 10 i.u./g.

Atlantic (grey) seal (Halichoerus grypus)

A single specimen weighing 59·5 kg., and estimated to be 18 months old, was shot by a fisherman off the Pembrokeshire coast in October 1946. Blubber (mean thickness, 3·4 cm.) from the same specimen was forwarded to Prof. T. P. Hilditch, Department of Industrial Chemistry, University of Liverpool, for a study of its composition (Hilditch & Pathak, 1947). The liver, which weighed 1·81 kg., contained 4 % of fat. The vitamin A content of the different portions analysed is shown in Table 8.

Table 8. *Distribution of vitamin A in organs of an unmated Atlantic seal killed October* 1946

Organ	i.u./g.
Liver	1,550
Liver oil	38,750
Lung	2·5
Left kidney	14·5
Right kidney	17·0
Suprarenals	Nil
Spleen	0·45
Subcutaneous fat	3·58

The total vitamin A reserve of the liver was calculated to be $2{\cdot}8 \times 10^6$ i.u.

Common seal (Phoca vitulina)

Vitamin A estimations were carried out on tissues of two of a total of four specimens recovered from the Wash on a field trip made in July 1947. These two animals were a very young female (estimated as being not more than 2–3 weeks of age, blubber thickness 2·5 cm.), and an immature male (estimated as not more than 4–6 weeks of age). The results are shown in Table 9.

Table 9. *Distribution of vitamin A in organs of two young common seals killed in July* 1947

Organ	i.u./g.: Very young female	i.u./g.: Immature male
Liver	12·0	90·0
Liver oil (8 %)	15·0	180·0
Lung	Nil	0·6
Kidney	Nil	0·9
Suprarenal	Nil	Nil
Spleen	Nil	Nil
Subcutaneous	Nil	Nil

SUMMARY

1. The weight, vitamin A content and fat content of the livers of sixty-four hooded seals (*Cystophora cristata*), 145 Greenland seals (*Phoca groenlandica*), one Atlantic seal (*Halichoerus grypus*), and two common seals (*Phoca vitulina*) were determined.

2. In *Cystophora cristata* and *Phoca groenlandica* from northern waters during the breeding season (March to late May) the concentration of vitamin A reached very high levels in mature specimens, up to 24,000 i.u./g. (mean 3,004 i.u./g.) in *Cystophora cristata*, and up to 15,000 i.u./g. (mean 3,441 i.u./g.) in *Phoca groenlandica*. The mean liver vitamin A content of four specimens of *Cystophora cristata* taken in the pack-ice east of Greenland in July 1948, was only 388 i.u./g.

3. The liver vitamin A content of the single specimen of *Halichoerus grypus* recovered from British waters fell within the range of the northern series, but those from the two young specimens of *Phoca vitulina* from the Wash were of much lower order.

4. There did not seem to be any correlation among mature specimens of any species between age, sex or thickness of the blubber and liver vitamin A.

5. The liver fat content in all species appeared to be high (8–10 %) in very young specimens, falling to between 2 and 4 % in mature animals.

6. Only relatively small amounts of vitamin A were found in the other tissues examined.

Our thanks are due to the Royal Norwegian Ministry of Commerce for financing the scientific expedition to the Newfoundland sealing grounds, and to the Dunn Nutritional Laboratory, Medical Research Council and University of Cambridge, for leave of absence of one of us to participate in the expedition; to Dr L. Harrison Matthews for his encouragement and valuable co-operation in the collection of material; and to Prof. A. N. Worden and Dr Thomas Moore for their interest in this work.

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A Method for the Estimation of Micro Amounts of Amino Nitrogen and its Application to Paper Partition Chromatography

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Since the introduction of paper partition chromatography (Consden, Gordon & Martin, 1944) it has been possible to separate completely many amino-acids and peptides from complex mixtures. This has focused attention on the need for quantitative methods for estimation of micro amounts of these substances after their separation on paper chromatograms. The method of Pope & Stevens (1939) has proved of great use in the estimation of amino nitrogen at macro and semi-micro levels. Preliminary notes of its use in a modified form for determining micro amounts of amino-acids separated by single-dimensional paper chromatography have already been given (Woiwod, 1948*a*, *b*). The present paper gives fuller details of the method, together with some preliminary results.

In the method of Pope & Stevens (1939) amino and 'peptide amino' nitrogen are estimated by allowing a suspension of copper phosphate to react with the material being investigated at pH 9·3, and, after filtration, the copper in the soluble complexes formed is estimated iodometrically. The terms 'amino nitrogen' and 'peptide amino nitrogen' just mentioned are used in this paper in the following sense. The groupings with which the copper phosphate reacts in proteins and their breakdown products are the α-amino (or imino) group of free amino-acids and the free amino groups of peptides and proteins, i.e. any not involved in peptide linkage. The nitrogen of the reacting group in free amino-acids is designated α-amino nitrogen and the nitrogen of the free amino groups in peptides and proteins is designated here as 'peptide' amino nitrogen. Little is known about the reactions of copper with peptide amino nitrogen. The limitation this imposes when interpreting results with partial digests of peptides and proteins is discussed later.

EXPERIMENTAL

The method of Pope & Stevens (1939) can be modified so that micro amounts of amino N can be estimated by using the diethyldithiocarbamate reaction (Callan & Henderson, 1929) to determine the Cu in the soluble copper complexes. The method consists of the following stages: (*a*) the amino-acid or peptide is allowed to react with a suspension of copper phosphate and the excess of the latter is then filtered off; (*b*) the Cu in a measured sample of the filtrate is determined by means of sodium diethyldithiocarbamate; (*c*) the Cu so determined is related to amino or peptide N, or weight of amino-acid or peptide, by means of a standard curve or use of a factor.

The ratio, *F*, of weight of α-amino N to weight of Cu reacting has been determined for a number of amino-acids by this method (Woiwod, 1948*b*). The copper phosphate suspension of Pope & Stevens (1939) was used in the preliminary work, but its use had several disadvantages at micro levels. These were: (*a*) a considerable deviation of the value of *F* from the theoretical value of 0·44 for complexes of the type A_2Cu was observed with most amino-acids, and the ratio was not constant from acid to acid; (*b*) the relationship between α-amino N and Cu was not linear; (*c*) the reagent did not keep well and needed to be freshly prepared for each set of determinations; (*d*) the reaction had a large temperature coefficient; and (*e*) the blank value given by the reagent was large.

By omitting the $Na_2B_4O_7$ and adding Na_2HPO_4 to the Cu reagent a linear relationship between amino nitrogen and copper over a considerable concentration range was obtained, and the value of *F* for many amino-acids was much nearer the theoretical value of 0·44. Thus it was possible to use this

factor for converting copper to amino N in comparative work not requiring the highest accuracy, and with mixtures of amino-acids such as complete hydrolysates of protein. The elimination of $Na_2B_4O_7$ also increased the stability of the reagent, which was then no longer affected by temperature, and blanks were low and consistent. It was, however, not possible to obtain the same value of F for all amino-acids. Mixing the components of the new reagent at room temperature gave a copper phosphate suspension the pH of which slowly increased on standing. At the same time the factor F obtained with standard amino-acid solutions slowly decreased, and with glycine most nearly approached the theoretical value of 0·44 when the reagent had stabilized. This stabilization was assisted by heating; refluxing the reagent for 1 hr. and leaving to 'age' 24 hr. before use gave a satisfactory final product. Such a reagent has been used over a period of 1–2 months with no apparent change in properties when tested with a standard glycine solution. For determining amounts of α-amino N of the order of 1 mg./ml. and determining Cu iodometrically the original reagent of Pope & Stevens (1939) was more satisfactory as it gave a greater uptake of Cu/mol. of amino N than the modified reagent.

METHOD

Apparatus. All glassware must be thoroughly cleaned to remove traces of Cu and a routine of cleaning rigidly followed if blanks are to be minimal and consistent. After use all glassware should be washed first with soap and water, then rinsed and transferred to a Pyrex glass vessel to soak overnight in N-HCl. After soaking, all tubes are tested with an acid solution of sodium diethyldithiocarbamate, rinsed with distilled water (less than 0·02 μg. Cu/ml.) and dried, and funnels are rinsed with distilled water.

Distilled water. All reagents are prepared with good grade distilled water (less than 0·02 μg. Cu/ml.). Where glass-distilled water is necessary its use is mentioned in the text. The following reagents are required:

Cupric chloride. Dissolve 27·3 g. $CuCl_2.2H_2O$ (A.R.) in boiled distilled water and make up to 1 l.

Trisodium phosphate solution. Dissolve 25·6 g. Na_2HPO_4 (A.R.) in 500 ml. boiled distilled water, add 180 ml. N-NaOH and make up to 1 l. with boiled distilled water.

Disodium hydrogen phosphate. Dissolve 25·6 g. Na_2HPO_4 (A.R.) in boiled distilled water and make up to 1 l.

Sodium diethyldithiocarbamate. Dissolve 2·0 g. in 100 ml. distilled water and filter. This solution precipitates slowly on standing and is refiltered immediately before use.

Standard copper solution. Dissolve 3·928 g. $CuSO_4.5H_2O$ (A.R.) in boiled glass-distilled water, add 1 ml. concentrated H_2SO_4 (A.R.) and make up to 1 l. This solution is diluted 1 in 10 with glass-distilled water for use. One ml. of this diluted solution contains 100 μg. Cu.

Copper phosphate suspension. The $Na_3(PO_4)_2$ solution (1 vol.) is added to 1 vol. of the $CuCl_2$ solution and well mixed. The Na_2HPO_4 solution (4 vol.) is then added, mixed, and the mixture boiled under reflux for 1 hr. The resulting copper phosphate suspension is allowed to 'age' for 24 hr. before use.

Preparation of standard copper curve

All water used in the preparation of the standard curve should be glass distilled. Volumes of the standard copper solution containing between 1 and 100 μg./Cu are pipetted into 5 ml. distilled water, then 0·1 ml. sodium diethyldithiocarbamate solution is added and the volume made up to 10 ml. with glass-distilled water. The yellow solution is shaken for 15 sec. with 10 ml. amyl alcohol (A.R.), centrifuged to clear the amyl alcohol layer and the optical density of this layer determined by means of a Spekker absorptiometer using blue glass filters no. 7 (maximal transmission at about 440 mμ.). The curve relating Cu to optical density is linear up to 6·0 μg. Cu/ml.

Preparation of standard amino-acid or peptide curve

For the highest accuracy it is necessary to prepare a standard curve for each specific amino-acid or peptide. These curves are reproducible and once prepared only need checking from time to time with a standard solution. Glass-distilled water is used in the preparation of the standard curve. The standard amino-acid or peptide solution is added by means of a Trevan microsyringe (Trevan, 1925) to 2·5 ml. of the Na_2HPO_4 solution, the added volume being 0·1 ml. or less. Not more than 50 μg. α-amino N or 25 μg. of peptide amino N should be added. After thorough mixing, 2·5 ml. of the copper phosphate reagent are added; the mixture is well shaken, allowed to stand for 30 min. and filtered through pleated papers (Whatman no. 42, 9 cm.) which prevent the copper phosphate suspension 'creeping'. The filtrate (2 ml.) is pipetted into 8 ml. of glass-distilled water in a centrifuge tube, 0·1 ml. of the diethyldithiocarbamate solution is added, mixed and stood for 10 min. to allow the reaction to proceed to completion. The solution is extracted with 10 ml. amyl alcohol with shaking for at least 15 sec., followed by centrifugation to clear the amyl alcohol layer. The optical density of the yellow solution is determined and the values recorded as μg. copper by means of the standard curve. A blank determination is carried out in triplicate at the same time, the procedure being identical except that an equal volume of glass-distilled water is added instead of the solution containing the amino N. The blank value is subtracted and the corrected Cu value obtained is multiplied by the dilution factor. Thus a standard curve is obtained covering the range 1–50 μg. of α-amino N or 1–25 μg. peptide amino N/5 ml.

Estimation of amino nitrogen

The procedure is the same as for the preparation of the standard curve, the unknown solution being added in place of the standard amino-acid solution. If it is not possible to keep the volume added to 0·1 ml., any convenient volume may be used, provided a standard curve is prepared using the same volume. Progressive dilution will lead, however, to a falling off in the copper uptake and a loss of sensitivity.

RESULTS

A preliminary survey of some 25 amino-acids and peptides has shown that alanylglycine, histidine, hydroxyproline, aspartic acid, serine, threonine, valine, leucine, isoleucine, glutamic acid, methionine, asparagine, glycine, tyrosine, arginine, phenylalanine, cystine, cysteine, tryptophan, glutamine, α-alanine, proline, lysine and ornithine all react satisfactorily with the reagent (Woiwod, 1948*a*, *b*). Table 1 gives a list of average F values obtained with

some further amino-acids and peptides including 'pantonine' (α-amino-ββ-dimethyl-γ-hydroxybutyric acid; Ackermann & Kirby, 1948; Holly, Barnes, Koniuszy & Folkers, 1948). It was not possible to examine in detail the behaviour of all these substances, but readings were made at 10 and 30 μg. levels of α-amino nitrogen or 5 and 15 μg. of peptide

Table 1. *Ratio (F) for some amino-acids and peptides of weight of α-amino or peptide amino nitrogen to weight of copper reacting*

Compound	Average *F* value
αγ-Diaminobutyric acid	0·25
α-Amino-*n*-butyric acid	0·43
α-Amino-ββ-dimethyl-γ-hydroxybutyric acid	0·46
Glycylglycine	0·22
Diglycylglycine	0·23
Triglycylglycine	0·24
γ-Aminobutyric acid	No reaction
4-Aminobutane-1-carboxylic	No reaction
5-Aminopentane-1-carboxylic	No reaction

amino nitrogen, and an average value for the factor *F* obtained from the slope of the curve drawn through these values. In most instances this curve passed through the origin, indicating a linear relationship between copper and nitrogen at least up to 30 μg. of α-amino nitrogen. At micro levels of amino nitrogen those acids which normally form sparingly soluble copper complexes, such as cystine, methionine, leucine and phenylalanine, all react well, presumably because the solubility product of their copper salts is not exceeded.

A more detailed study has been made of glycine, valine and leucine. These give linear curves up to 30 μg. of α-amino nitrogen, but deviate from linearity between 30 and 50 μg. α-amino nitrogen. Excessive dilution of the sodium phosphate solution is inadvisable as the amount of copper reacting/mole of α-amino nitrogen is affected by the strength of this solution. The effect is shown in Table 2, where

Table 2. *The effect of* Na_2HPO_4 *concentration on the amount of copper reacting with glycine and lysine*

Amino-acid	Na_2HPO_4 (% saturation)	Copper uptake (μg. Cu/2 ml.)	*F* value
Glycine	8	6·4	0·62
(10 μg. α-amino N/5 ml.)	32	7·9	0·51
	60	9·7	0·41
	100	11·4	0·35
Lysine	8	4·8	0·83
(10 μg. α-amino N/5 ml.)	32	5·7	0·70
	60	7·5	0·53
	100	8·8	0·45

increasing strengths of disodium hydrogen phosphate solution up to saturation give increasing copper uptake with standard solutions of glycine and lysine. Ten μg. amounts of α-amino nitrogen were used in every case, the figures in the third column being the copper value in a 2 ml. sample of the filtrate. The blank copper value also rises slowly with increasing phosphate concentration.

It seems unlikely that any adjustment in the composition of the reagent will give the theoretical value of 0·44 with all acids. The final composition chosen is one which gives this value with glycine. This compound was used for much of the exploratory work. Most of the amino-acids tested give values which lie fairly close to this theoretical value, suggesting the formation of complexes of the type A_2Cu. A number of α-amino-acids, however, behave in exceptional ways. Lysine and ornithine both react less readily than other amino-acids, giving an average *F* value of about 0·5, whereas histidine and αγ-diaminobutyric acid have approximately the same *F* value as peptides (0·22), indicating the formation of a complex of the type *A*Cu. Mono-amino-acids with amino groups at other than the α-position show little or no reaction with copper phosphate. β-Alanine reacts very slightly and γ-aminobutyric, 4-aminobutane- and 5-aminopentane-1-carboxylic acids do not react at all. This failure of amino-acids with the amino group at other than the α-position to form copper salts has enabled Dent (1948) to separate such acids from α-amino-acids by running the copper salts of the mixed acids on two-dimensional paper chromatograms. A mono-amino-acid with the amino group at a position other than α will not be affected by copper phosphate treatment, whereas the α-amino-acids will fail to appear in their usual positions and run to one side of the paper chromatogram. All the peptides so far tested have given values of *F* very near that expected for the formation of a complex *P*Cu.

Application to paper chromatography

The copper phosphate reagent reacts satisfactorily with amino-acids separated by paper chromatography (Woiwod, 1948*a*, *b*) and has been used to determine the ratio of leucine to threonine in a sample of polymyxin A (Jones, 1948). The amino-acid is first located on the chromatogram by inspection in ultraviolet light; this involves no destruction of the acids or peptides. The paper is dried for 3–4 hr. at 80° (Woiwod, 1949), and then examined in the dark under illumination from an ultraviolet lamp. The amino-acids and peptides appear as light blue fluorescent spots on a dark purple fluorescent background.

A square of filter paper (approx. $1\frac{1}{2} \times 1\frac{1}{2}$ in.) with the amino-acid or peptide spot on it is cut into strips about $\frac{3}{4} \times \frac{1}{4}$ in. These are dropped as a bundle into a dry test tube $6 \times \frac{5}{8}$ in., 2·5 ml. of Na_2HPO_4 solution is added and the tube left to stand 30 min. with occasional swirling. Copper phosphate suspension (2·5 ml.) is then added and the mixture, after standing a further 30 min., is filtered. The filtrate is treated as described for the determination of amino N. A blank square of paper of the same size is cut from the paper and carried through an identical series of operations. The Cu value obtained after correcting for the blank is converted

to N or weight of material by means of a standard curve. The blank consists mainly of soluble copper from the copper phosphate suspension and is normally about 1·0 μg. copper/ml. of filtrate. A square of Whatman (nos. 1 or 4) paper $1\frac{1}{2} \times 1\frac{1}{2}$ in. gives no readable copper colour when tested without the addition of copper phosphate.

Early work with glycine and valine using *n*-butanol with ammonia as the solvent give recoveries of amino N between 90 and 95% after chromatography (Woiwod, 1948*a*). After further development of the copper reagent, recoveries from chromatograms were re-investigated. The solvent used was *n*-butanol-acetic acid (Partridge, 1948) run on Whatman no. 4 paper, this combination of solvent and paper being used for routine single-dimensional chromatography in this laboratory. Glycine, valine and leucine were studied. Ten spots of each substance were run for 16 hr., the spots located by ultraviolet light and the amino N determined therein. The recoveries, based on the copper in a 2 ml. sample of filtrate, are shown in Table 3, together with the standard deviation from the mean of the ten results at each level. The results indicated that the percentage recovery decreased with increasing R_F values. 'Collidine' gave a similar result with five spots each of glycine and leucine.

If the loss of amino N increases with the distance the spots travel, then decreasing recoveries should be obtained when the same substance is run on the chromatogram for increasing times. This is the case with glycine (Fig. 1). Samples containing 20 μg. α-amino N were run for various times on Whatman no. 4 paper with *n*-butanol-acetic acid as solvent, the paper removed, dried and determinations of amino N made. There was a progressively poorer recovery with increase in the distance the amino-acid had travelled from the starting line. Little work has yet been done with recoveries from two-dimensional paper chromatograms. It was thought, however, worth seeing whether the method could be applied to such paper chromatograms. A mixture of glycine, valine and leucine was run in duplicate first with *n*-butanol-acetic acid and then with phenol as solvents, in that order. After 16 hr. in each direction on Whatman no. 4 paper, the spots were located by ultraviolet light and the amino N determined. The recoveries are shown in Table 3. Considerably greater losses occurred with the two-dimensional paper chromatograms than in the single-dimensional runs. The losses, again, were greatest with the faster running compounds. It was thought that adsorption was the most likely cause of the losses. It was noted, however, that with single-dimensional paper chromatograms a 'tramline' of fluorescence often extended right to the solvent front. It may therefore be that failure of the acids to reach equilibrium with the solvent in the moving phase is partly responsible for the losses.

The rather large losses of amino N on paper chromatograms, particularly with faster running amino-acids, probably accounts for the larger quantity of amino N needed to obtain good two-dimensional paper chromatograms. The amino-acids, besides being depleted during the run, are also dispersed over three to four times as great an area as compared with a single-dimensional paper chromatogram. For this reason more satisfactory two-dimensional paper chromatograms are obtained when the starting spot is kept as small as possible, preferably 0·1 in. in diameter, or less (Woiwod, 1949).

Table 3. *Recovery of amino nitrogen from chromatograms of single amino-acids on Whatman no. 4 paper*

Solvent	Amino-acid	Approx. R_F value	Before running (μg. Cu/2 ml.)	Recovery (μg. Cu/2 ml.)	Standard deviation (μg. Cu/2 ml.)	Recovery (%)
Single-dimensional paper chromatograms run 16 hr.						
n-Butanol-acetic acid	Glycine	0·2	18·6	17·7	0·3	95·1
	Valine	0·4	28·8	26·1	0·6	90·7
	Leucine	0·6	21·6	17·6	0·8	81·5
'Collidine'	Glycine	0·1	18·6	17·5	—	94·1
	Leucine	0·4	21·6	17·6	—	81·5
Two-dimensional paper chromatograms run 16 hr. each way						
n-Butanol-acetic acid and phenol	Glycine	—	18·6	16·1	—	86·5
	Valine	—	28·8	20·6	—	71·6
	Leucine	—	21·6	13·8	—	63·9

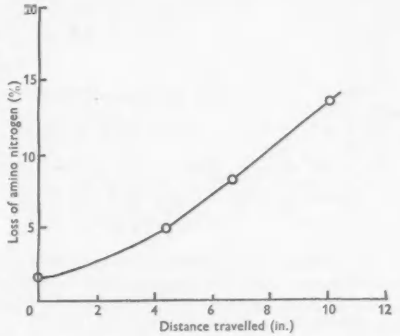

Fig. 1. Curve illustrating the progressive loss of amino N with increasing distance travelled from starting line. Single-dimensional paper chromatogram run with *n*-butanol-acetic acid on no. 4 Whatman paper. Original glycine concentration equivalent to 20 μg. α-amino N.

The destruction of amino-acids by ninhydrin on paper chromatograms

The usual procedure for locating amino-acids on paper chromatograms is to spray with a solution of ninhydrin and then to heat. Coloured spots are obtained which indicate the position of the various amino-acids and peptides. An investigation, by means

of the copper reagent, of the breakdown of amino-acids occurring during ninhydrin colour development, indicated that considerable quantities of amino nitrogen remained after colour development was apparently complete. Before the copper reagent could be applied to ninhydrin-sprayed papers, however, a method had to be found for blocking the reaction between excess ninhydrin on the paper and copper phosphate. The addition of 4·0 g./l. of sodium borate to the disodium hydrogen phosphate solution completely inhibited the reaction. That there was not complete destruction of amino-acids by ninhydrin was noted by Work (1948) who rechromatographed a spot already fully developed by ninhydrin and was able to obtain a coloured spot in a new position after respraying with more ninhydrin and heating. We have confirmed this observation. With glycine it has been found that after spraying with ninhydrin, heating for increasing times and estimating the residual glycine with copper phosphate reagent, there is a rapid initial destruction of amino nitrogen and then breakdown practically ceases. Respraying with ninhydrin solution and reheating causes little further destruction, nor is extra colour development obtained. This behaviour, illustrated in Fig. 2, would

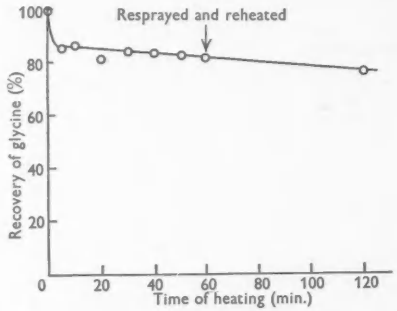

Fig. 2. Residual glycine, by copper phosphate estimation, on no. 4 Whatman paper after spraying with 0·1 % ninhydrin in chloroform and heating. Original glycine concentration equivalent to 20 μg. α-amino nitrogen.

be consistent with the formation of a substance inhibiting the reaction between ninhydrin and glycine on paper. The ninhydrin reaction is undoubtedly complex, as solutions of the coloured spots give a number of coloured bands on an alumina column, and it seems probable that among the products of the reaction such an inhibitor could be present. It was suggested (Woiwod, 1948*a*) that the failure of ninhydrin to destroy more than a portion of the amino-acid in a spot on paper might enable acids to be located by this reagent before quantitative estimation. The difficulty in assessing the degree of breakdown, however, led to abandonment of the method in favour of using ultraviolet light for locating the spots.

DISCUSSION

The copper phosphate reagent described here can be used to determine both the α-amino group of amino-acids, and the free amino groups in polypeptides and proteins which have been called 'peptide amino' groups in this paper. Although two conversion factors are therefore necessary, this raises no problems when determinations are made on solutions of isolated amino-acids or peptides, as the necessary conversion can in most cases be achieved by means of standard curves prepared beforehand. With mixtures of α-amino-acids and peptides, however, such as those in partial hydrolysates of proteins, it is not possible to say with certainty what factor should be used for converting copper into amino nitrogen.

Pope & Stevens (1939) followed the digestion of fibrin by trypsin and showed that the results they obtained with their copper reagent agreed with those given by the gasometric Van Slyke method. A conversion factor which assumed that 1 mole of copper was equivalent to 2 moles of amino nitrogen was used. It may be that very long peptide chains reacted under their conditions in such a way that this factor was a fair approximation. However, the few peptides tested at micro levels (Table 1) react as if a complex PCu was formed. By the ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941) it is possible to determine and to correct for free α-amino nitrogen in a complex protein digest, and hence to obtain the peptide amino nitrogen, assuming a factor of 0·22 to hold in all circumstances when converting copper into peptide amino nitrogen. This assumption may not be justified in view of the lack of knowledge existing about the reaction of copper with peptide amino nitrogen. Though the ϵ-amino group of lysine appears to be inactive in the free acid no information is available as to its reaction when the α-amino group is in peptide linkage. Similarly, it is by no means certain how the iminazole group of histidine will react when its α-amino group is in peptide linkage. In practice, however, these limitations are not found to detract from the usefulness of the copper reagent in following the rate of hydrolysis of a protein, when an arbitrary factor of 0·44 for converting copper to amino nitrogen is used. The presence of such acids as histidine or $\alpha\gamma$-diaminobutyric acid in large amounts in peptides or proteins makes it difficult to assess the degree of breakdown of such materials, as both these amino-acids form complexes of the type ACu, and amino-nitrogen figures in excess of the total nitrogen figures can be obtained if the factor 0·44 be used. By plotting amino nitrogen against

time a guide to the completeness of the digestion can be obtained although the figures have no absolute significance. Attention is drawn to these points as ignorance of these effects might lead to faulty conclusions being drawn from experimental results.

The failure to obtain a theoretical conversion factor with many amino-acids is at first sight puzzling, since it appears in some instances as if more copper were incorporated than the formation of the complex A_2Cu would allow. Borsook & Thimann (1932), however, showed that a number of copper complexes of both glycine and alanine can be obtained and can co-exist in solution; similar behaviour with other acids could account for the anomalous results obtained in the present work. Furthermore, it is not certain that the soluble copper salts estimated by the present method correspond to the isolated copper salts of the amino-acids.

SUMMARY

1. A method is described for determining micro amounts of α-amino nitrogen (1–50 μg.) and peptide amino nitrogen (1–25 μg.).

2. The method has been applied to single- and two-dimensional paper chromatograms of a number of α-amino-acids and an evaluation made of the losses involved during chromatography.

3. The destruction of amino-acids on heating the spots on paper chromatograms developed with ninhydrin has also been studied quantitatively. It is suggested that a decomposition product makes the reaction self limiting.

I am indebted to Dr C. E. Dent for samples of the non-α-amino-acids investigated. I also wish to thank Mr R. Knight for valuable technical assistance.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors

SUBSTANCES ABLE TO REPLACE OLEIC ACID FOR THE GROWTH OF *CORYNEBACTERIUM* 'Q' WITH A NOTE ON A POSSIBLE METHOD FOR THEIR MICROBIOLOGICAL ASSAY

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Forty years ago, Fleming (1909) observed the stimulatory effect of oleic acid on the growth of *Corynebacterium acnes*, but it is only recently that the essential nature of long-chain unsaturated fatty acids for the growth of certain organisms has been firmly established. It is now generally accepted that linoleic acid is essential for the proper nutrition of rats (see Hansen & Burr, 1946), while Fraenkel & Blewett (1947) have shown that it (or linolenic acid) is necessary for normal development of the moth *Ephestia kuehniella*. Benham (1941) has reported that oleic acid is essential for the growth of the fungus, *Pityrosporum ovale*, while, amongst bacteria, oleic acid has been found necessary for the growth of some strains of *Corynebacterium diphtheriae* from a small inoculum (Cohen, Snyder & Mueller, 1941), for *Clostridium tetani* (Feeney, Mueller & Miller, 1943), for *Cl. sporogenes* in the absence of biotin (Shull, Thoma & Peterson, 1949), and for the unidentified *Micrococcus* 'C' (Dubos, 1947). There have also been reports, of which the most accurate and complete are those of Williams & Fieger (1946)

and Williams, Broquist & Snell (1947), proving the necessity of oleic acid or linoleic acid for some strains of lactobacilli, usually only in the absence of biotin.

During experiments on *Haemophilus pertussis*, a chance contaminant was isolated which was found to have the property of symbiotically stimulating the growth of *H. pertussis* in nutrient broth and other media without blood (Pollock, 1948*a*, 1949). This organism proved, on further investigation, to be unable to grow in a medium of known composition without the addition of oleic acid. It was found to be an obligatory aerobic diphtheroid, fermenting glucose, but not lactose, sucrose, mannitol or dulcitol, and has been provisionally labelled *Corynebacterium* 'Q'. Its general properties and its clear-cut response to oleic acid suggested that it might be a suitable test organism for a study of the function and metabolism of long-chain fatty acids.

METHODS

Glassware. All glassware was thoroughly cleaned with hot chromic acid to remove traces of fat, and the 50 ml. Erlenmeyer flasks used for growth experiments were plugged with special fine glass wool (Fibreglass Ltd.) previously extracted with methanol and acid-cleaned (Pollock, 1948*b*).

Medium. 'Vitamin-free' casein hydrolysate (Ashe), supplemented by tryptophan, cystine, salts and growth factors (pH 7·6) as follows. Basal medium (final concentrations): casein hydrolysate (Ashe, 'vitamin-free'), 5·0; KH_2PO_4, 4·5 mg./ml.; NaOH, to pH 7·6; L-cystine, 0·0024; L-tryptophan, 0·0008; $MgSO_4$, 0·02; $FeSO_4$, 0·0015 mg./ml. Growth factors 'A': biotin 0·01; nicotinamide, 1·0; Ca pantothenate, 0·75; pyridoxine, 2·5; riboflavin, 1·2 aneurin; HCl, 0·5; *p*-aminobenzoic acid, 0·45; haemin, 2·0 μg./ml. Growth factors 'B': guanine sulphate, 11·0; inositol, 9·0; uracil, 5·5; adenine sulphate, 10·0; cytosine, 5·5; pimelic acid, 0·8; folic acid, 1·0 μg./ml.

All tests classified in Table 1 were done in a medium consisting of casein hydrolysate, salts and growth factors 'A'. For the accurate curves depicting the relationship between growth opacity and fatty acid concentration (Fig. 1) both groups of factors 'A' and 'B' were included in the basal medium.

This was sterilized by Seitz filtration; the haemin was first autoclaved and added after the rest of the medium had been sterilized.

Inoculum. One drop of a 1:100 dilution of a standard suspension of cells grown on tryptic meat agar (without added oleic acid) for 24 hr. and once washed with water. This was found to contain on an average 50,000 viable cells.

Incubation. Unless otherwise stated, cultures were incubated aerobically at 37°, and growth was recorded by eye after 1, 2 and 3 days as tr., tr. +, +, + +, etc. Flasks were left for 7 days before recording a negative result.

Fatty acids. Trivial and systematic names of fatty acids mentioned in the text are as follows: brassidic, *trans*heneicos-12-ene-1-carboxylic acid; dihydroxystearic, 8:9-dihydroxyheptadecane-1-carboxylic acid; dibromostearic, 8:9-dibromoheptadecane-1-carboxylic acid; erucic, *cis*heneicos-12-ene-1-carboxylic acid; elaeostearic, heptadeca-8:10:12-triene-1-carboxylic acid; elaidic, *trans*heptadec-8-ene-1-carboxylic acid; lauric, undecane-1-carboxylic acid; linoleic, heptadeca-8:11-diene-1-carboxylic acid; linolenic, heptadeca-8:11:14-triene-1-carboxylic acid; myristic, tridecane-1-carboxylic acid; oleic, *cis*heptadec-8-ene-1-carboxylic acid; αβ-oleic, heptadec-1-ene-1-carboxylic acid; palmitic, pentadecane-1-carboxylic acid; palmitoleic, pentadec-8-ene-1-carboxylic acid; petroselinic, heptadec-5-ene-1-carboxylic acid; ricinoleic, 11-hydroxyheptadec-8-ene-1-carboxylic acid; stearic, heptadecane-1-carboxylic acid.

The fatty acids used were the purest that could be obtained. Palmitic and stearic acids and the first samples of linoleic and linolenic acids were gifts from Dr W. T. J. Morgan; lauric, myristic, petroselinic, elaeostearic and the second samples of linoleic and linolenic acids were supplied by Prof. T. P. Hilditch, the four latter as methyl esters. These esters were hydrolysed by heating with N-NaOH for 30 min. at 100° under N_2. Pure oleic acid was obtained by distilling a commercial sample (Hopkin and Williams 'Purified' grade), followed by purification through dibromostearic acid (Holde & Gorgas, 1926) and redistillation *in vacuo*. Methyl palmitoleate was isolated from distilled sperm oil fatty acids. The methyl esters of the liquid acids prepared by the Pb salt method (Hilditch, 1940) were fractionated *in vacuo* using an 8 in. Fenske column fitted with a heated jacket and reflux ratio head. The fraction boiling at 138–139°/1·6 mm. had an I_2 number of 102 (Trappe, 1938), and gave an acid with an equivalent of 256. The dihydroxystearic acids were prepared by the methods of Wittcoff, Moe & Iwen (1948) and Scanlan & Swern (1940). Elaidic acid was prepared by the method of Bertram (1936). Tween 80 (Atlas Powder Co.) was purified according to Davis (1947). Ricinoleic acid and erucic acid were purified by partition chromatography (Howard & Martin, unpublished).

All fatty acids were dissolved in the minimum amount of NaOH needed to give a stable solution or emulsion and added to the basal medium without further treatment, apart from sterilization by autoclaving at 15 lb. pressure for 20 min.

In experiments where growth was measured turbidimetrically, the Spekker absorptiometer was used, with neutral grey filter H 108, and the concentration of fatty acid added plotted against the opacity of the culture (expressed as $\log I_0/I$). The relationship between opacity and dry bacterial weight was established by making serial dilutions of a washed suspension containing a known weight of cells in the medium used for growth, and plotting a standard curve. The dry weight of organisms was measured by drying a suspension of cells twice washed with distilled water in an oven at 110° to constant weight.

RESULTS

Effects of different fatty acids

If strict precautions were taken to eliminate all traces of extraneous fat, no visible growth ever occurred in the basal medium. Attempts to simplify this medium were not very successful, although it was found that satisfactory growth occurred in a mixture containing eighteen amino-acids, Fe^{++}, Mg^{++}, and eight 'A' growth factors (see 'Methods') together with 10 μg./ml. oleic acid. After this confirmation that 'Q' would grow as well in a medium of known composition as in the casein hydrolysate,

and since no growth occurred in the latter in the absence of oleic acid, it was decided to use the casein hydrolysate as a basis for subsequent tests rather than the amino-acid mixture, for the sake of economy.

Table 1. *Ability of different long-chain fatty acids to support the growth of* Corynebacterium '*Q*'

(Medium: casein hydrolysate + growth factors (see 'Methods'); incubation at 37°, aerobically; inoculum, about 50,000 cells.)

Acid tested	Concentration (μg./ml.)	Growth after 1 day	2 days	3 days
Oleic	25	tr.	+	+ + +
	5	+	+ +	+ +
	1	tr. +	tr. +	+
Elaidic	25	+	+ +	+ + +
	5	+	+	+ +
	1	tr. +	tr. +	+
Petroselinic	25	? tr.	tr.	+ + +
	5	–	tr. +	+
	1	–	tr.	tr. +
Palmitoleic	25	–	–	–
	5	+ +	+ +	+ + +
	1	+	+	+
Linoleic	25	–	+ +	+ + +
	5	–	+ +	+ +
	1	tr. +	+	+
Linolenic	25	–	–	–
	5	–	+ +	+ +
	1	tr. +	+	+
Tween 80 (purified)	25	tr.	+ +	+ + +
	5	tr.	+	+
	1	–	–	tr.

Lauric	Brassidic	No growth up to 7 days, at conc. of 1, 5, and 25 μg./ml.
Myristic	αβ-Oleic	
Palmitic	*cis*Dihydroxystearic	
Stearic	*trans*Dihydroxystearic	
Ricinoleic	*cis*Dibromostearic	
Erucic	*trans*Dibromostearic	

The results are summarized in Table 1. For oleic, elaidic, petroselinic, linolenic, linoleic and palmitoleic acids, the relationship between concentration of fatty acid in the medium and total yield of organisms in dry weight after incubation for 3 days is expressed in the form of curves based on accurate opacity measurements (Fig. 1). None of the acids which failed to promote growth had any detectable inhibitory effect at a concentration of 25 μg./ml. in the presence of 10 μg./ml. oleic acid.

Three different preparations of oleic acid and two each of linoleic and linolenic acids gave substantially the same results. It will be noticed that the higher concentrations of palmitoleic, linoleic and linolenic acids were markedly inhibitory, and it was first supposed that this might be due to some toxic impurity present in relatively small amount, particularly since the first samples tested of linoleic and linolenic acids were nearly 20 years old. However, the second samples of these two acids, which were freshly prepared, kept as their methyl esters and tested on the same days as the hydrolysis to the free acid, gave exactly the same results as the original preparations. This 'double action' effect which characterizes the action of unsaturated fatty acids on a number of different bacterial species (see Pollock, 1949) is much more marked in the case of oleic acid acting on lactobacilli (Williams *et al.* 1947).

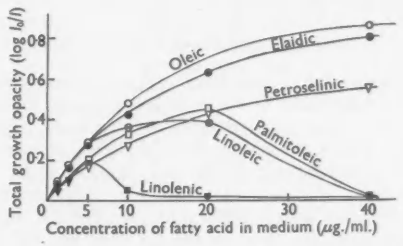

Fig. 1. Relation between total final growth of *Corynebacterium* 'Q' and concentration of different fatty acids in the medium. Basal medium: casein hydrolysate plus growth factors (see 'Methods'); incubation, aerobically at 37° for 3 days; inoculum, about 50,000 cells.

It can be seen that the quantitative response to the active compounds is nearly the same, although petroselinic acid is not apparently quite so active as oleic acid; while with palmitoleic, linoleic and (especially) linolenic acid the picture is complicated by the superposition of an inhibitory effect on the growth-promoting one. The most striking result is that shown by the 'unnatural' elaidic acid which gave almost exactly the same yield of cells as its isomer, oleic acid. However, with petroselinic acid it was observed that the rate of growth was considerably slower than with oleic acid (Table 1).

All saturated fatty acids tested were without effect. The action of purified Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate) shows conclusively that the organism can utilize the oleate in this esterified form, the slight delay in growth compared to free oleic acid possibly being due to the need for preliminary hydrolysis, or to slower diffusion through the cell wall. Utilization of purified Tween 80 by certain oleic acid-requiring lactobacilli has also been reported by Williams *et al.* (1947). Samples of ricinoleic acid and erucic acid gave, when first tested, slight growth in flasks containing the higher concentrations. This, however, was shown to be due to some residual impurity for, after passing these acids down a chromatographic column (Howard & Martin, unpublished), neither compound gave any growth whatever up to 25 μg./ml.

α-Elaeostearic acid was tested on several occasions, but the results were variable and have not been recorded. It was discovered that the iodine number of elaeostearic acid fell from 260 to 142 when the methyl ester was hydrolysed by heating 0·5 g. at 100° with 2N-NaOH, and continued to fall during incubation in M/30 phosphate buffer at pH 7·6 and 37°, reaching 32 in 5 days. Tests with this acid are therefore meaningless. This destruction of elaeostearic acid under aerobic conditions was also observed by Kodicek & Worden (1946).

Microbiological assay of oleic acid

The relative lack of specificity in the response of *Corynebacterium* 'Q' to fatty acids clearly reduces its value as an assay organism for oleic acid. Nevertheless, on occasions, the microbiological estimation of minute amounts of oleic acid (known to be the sole fatty acid present), or of oleic acid-like substances, has been of some value (see Pollock, 1949), particularly as there is yet no chemical method available. The basal medium employed was the same as that used in the ordinary growth experiments, with the addition of 'B' group of growth factors; and the technique of inoculation, incubation, etc. was similar. Satisfactory duplicates were obtained by incubating for 3 days in the usual way, but a linear response between concentration of fatty acid added and dry weight of cells produced was not obtained unless the flasks were shaken to promote maximal aeration. Table 2 gives the values obtained, with their standard deviations, up to a concentration of 10 μg./ml. of added oleic acid. It appears that concentrations of oleic acid between 2·0 and 10·0 μg./ml. could be assayed with an error of not more than 15%. Slight variations in the relationship between growth and concentration of oleic acid were found to occur from day to day. Flasks containing at least two concentrations of oleate were therefore set up and inoculated at the same time as the test, so that a fresh standard curve could be prepared each time. Tryptic meat broth and also 2% peptone water were found to be almost as satisfactory as the fat-free casein hydrolysate medium for such estimations, although, of course, allowance has to be made for the slight growth (equivalent to an oleic acid content of about 0·4 μg./ml. in both cases) which occurs in the basal medium without added oleic acid.

Table 2. *Microbiological assay of oleic acid with* Corynebacterium '*Q*'

(Flasks were incubated at 37° for 3 days on a mechanical shaker. Basal medium: 'Vitamin-free' casein hydrolysate with sixteen growth factors (see 'Methods'). Inoculum: about 50,000 cells.)

Oleic acid added (μg./ml.)	No. of flasks	Mean yield of cells (μg. dry wt./ml.)	Standard deviation
2·0	8	61	±3·6 (6%)
5·0	8	144	±4·2 (2·9%)
10·0	5	253	±6·8 (2·7%)

A crude biological extract was prepared by heating a washed suspension of *Escherichia coli* for 30 min. at 100° and spinning off the cell debris. The supernatant liquid was found to have a growth-stimulating action for 'Q' corresponding to an oleic acid content of 41·5 μg./ml., and the added oleic acid could be estimated satisfactorily in the presence of this extract diluted 1:9 (see Table 3). However, the assay of oleic acid in the presence of substances such as undenatured serum albumin or soluble starch, both of which have a high combining affinity for oleic acid, was found to be unsatisfactory. It is clear that an attempt to assay unsaturated fatty acids in biological material of unknown composition would have to be preceded by an extraction of all lipid with some fat solvent, or the demonstration that added oleic acid could be satisfactorily estimated in its presence.

DISCUSSION

In general, the results reported here for *Corynebacterium* 'Q' confirm and extend those of Williams & Fieger (1946) and Williams *et al.* (1947) with lactobacilli. The range of compounds able to replace oleic acid is relatively narrow. At least one double

Table 3. *Effect of crude biological extract on assay of oleic acid with* Corynebacterium '*Q*'

(The crude extract used was an aqueous infusion of a boiled cell suspension of *Escherichia coli*.)

Extract present (ml.)	Oleic acid added (μg./ml.)	Yield of cells (μg. dry wt./ml.)	Total assay in terms of oleic acid (μg./ml.)	Oleic acid 'recovered' (μg./ml.)	Oleic acid 'recovered' (%)
0	0	0	~	~	—
0	5·6	158	~	~	—
0	2·2	86	~	~	—
0·25	0	31	1·0	~	—
0·25	5·0	180	5·8	4·8	96
0·25	2·0	113	3·3	2·3	115
1·0	0	145	4·7	~	—
1·0	5·6	300	9·7	5·0	90
1·0	2·2	226	7·3	2·6	118

bond appears to be absolutely essential, but its exact position is less important. In the C_{18} series the 5:6 position* (petroselinic acid) is nearly as satisfactory as the 8:9 (oleic acid), although $\alpha\beta$-oleic acid (1:2 position) is inactive. The configuration around the double bond is apparently not critical, since elaidic acid has the same activity as its *cis*-isomer, oleic acid. A single hydroxyl group, however, in the 11 position (ricinoleic acid), abolishes activity. Shortening the chain length by two methylene groups, leaving the double bond in the same position relative to the carboxyl group (palmitoleic acid), does not greatly reduce the activity, but the C_{22} acids, erucic and brassidic, with the double bond in the 12:13 position, are quite inactive.

The same relative lack of importance of the position of the essential double bond has been reported by Shull *et al.* (1949), who found that *cis*-vaccenic acid ($\Delta^{10,11}$ isomer of oleic) would completely replace oleic acid for the growth of *Cl. sporogenes* in the absence of biotin. These workers also found that the *trans* isomers of both oleic and vaccenic acids were active, only less so than oleic acid itself. For lactobacilli, too, elaidic acid has been reported (Williams & Fieger, 1946) to be able to replace oleic acid.

The linear relationship between growth and fatty acid concentration of the medium suggests that oleic acid and its analogues are used to form some essential constituent of the cell protoplasm. The figures already quoted show that oleic acid consistently supports the growth of almost thirty times its own weight of cells of *Corynebacterium* 'Q'. Nevertheless, it must be emphasized that knowledge of the functions and metabolism of fatty acids in bacteria is scanty. The possibility that the growth-promoting effect of oleic acid may be due to some physicochemical effect (e.g. on the permeability of the cell wall, as suggested by Kodicek, 1949), or purely to its acting as a specific source of energy, cannot be completely ruled out. It is also conceivable, although very unlikely, that the difference in growth-promoting effect between active and inactive fatty acids is due possibly to very great differences in the ease with which they may be absorbed through the cell wall. Moreover, the constancy of cell composition in relation to the quantity and quality of the nutrients provided is not, in general, so marked in the case of lipids as it is with protein and carbohydrate.

It is thus not easy to speculate on such questions as, for instance, whether elaidic acid is absorbed and utilized as such, or is transformed into oleic acid. The latter would appear more likely since elaidic acid has not yet been found to occur naturally in any organism. Sinclair (1935), however, has shown that it can replace up to 30% of the natural fatty acids in the phospholipins of liver and muscle if fed to rats, and Paul & McCay (1942) claim that guinea pigs will absorb (and probably 'utilize') 95% of the elaidic acid fed in the diet.

Clearly, such questions will not be finally answered until it is possible to investigate how far and in what way the fatty acid in the growth medium affects the fatty acid composition of the cells when grown. It is hoped to carry out further research along these lines, as well as to extend the range of compounds tested for their ability to replace oleic acid for growth of this organism.

* In this discussion the numeration is on the basis of Chemical Society nomenclature, in which —COOH is a substituent on $C_{(1)}$.

SUMMARY

1. A number of long-chain fatty acids have been tested for their ability to support the growth of an oleic acid-requiring diphtheroid bacterium (*Corynebacterium* 'Q') in a medium of known composition.

2. None of the saturated fatty acids tested (lauric, myristic, palmitic, stearic) was active. Of the unsaturated acids, linoleic, linolenic, palmitoleic, elaidic and petroselinic acids were roughly equivalent to oleic acid, although the first three were inhibitory in the higher concentrations. Erucic, brassidic, $\alpha\beta$-oleic and ricinoleic acids, however, were all quite inactive.

3. A possible method for the microbiological assay of oleic acid in concentrations between 2·0 and 10·0 μg./ml. has been outlined.

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Phospholipin Metabolism in Rabbit-liver Cytoplasm

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The recent work of Fishler, Entenman, Montgomery & Chaikoff (1943) and of Entenman, Chaikoff & Zilversmit (1946) demonstrated that liver is the principal tissue in the body concerned with the production and removal of plasma phospholipins. However, it is not known whether synthesis and decomposition of phospholipins take place uniformly throughout the liver cell or whether these functions are associated with a particular type of particle in the cell. The present work deals partly with the technique of liver-cell fractionation and partly with the study of the rates of renewal of phospholipins in liver-cell fractions. Hevesy (1945) showed that the rate of renewal of phospholipin phosphorus in liver nuclei is slower than in the cytoplasm. His observation is confirmed, and it is shown that there are at least three different rates of renewal in different fractions of the liver cytoplasm.

1. ANALYSIS OF LIVER FRACTIONS

There have been numerous attempts to fractionate liver into components which can be directly related to visible granules in the liver cell. Several methods, e.g. those of Marshak (1941–2), Dounce (1943), Mirsky & Pollister (1946), have been used to prepare liver-cell nuclei, while methods have been described by Claude (1946*a*), and more recently by Hogeboom, Schneider & Pallade (1948) for the fractionation of liver cytoplasm. Claude described the preparation of three fractions: (1) large granules, corresponding to the mitochondria and secretory granules of the histologist; (2) microsomes (small granules), composed of particulate elements of submicroscopic size; and (3) a supernatant, containing the particles and molecules which remain in solution after the first two fractions had been removed. Hogeboom *et al.* (1948) have criticized Claude's (1946*a*) procedure on the ground that the granules isolated in fraction 1 are not identical morphologically or in staining reactions with the mitochondria seen in the intact cell, and they have described a method of isolating these intact.

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MATERIAL AND METHODS

In an earlier (unpublished) series of experiments normal rabbit liver was fractionated by Claude's (1946*a*) procedure. The presence of glycogen, however, interfered seriously with the fractionation, as has also been observed by Claude. In subsequent experiments this effect was largely eliminated by withholding food from the animals the night before the removal of the liver.

In view of the evidence of Hogeboom *et al.* (1948) that the large granules prepared by the use of a concentrated solution of sucrose more closely resemble the mitochondria of the intact cell, their method (in a slightly modified form) was used for most of the present investigation. In our work the first consideration has been the purity of the fractions, necessarily at the expense of information about the amount of each present.

Fully grown (2·3–2·8 kg.) rabbits were used. They were anaesthetized with nembutal and ether, and after withdrawal of blood from the aorta, the liver was excised. All fractionation procedures were carried out at or near 0°.

Liver fractionation. Hogeboom *et al.* used 0·88 M-sucrose solution in their procedure. For the sedimentation of the large and small granules in a reasonably short time from a solution of such high density, centrifugal forces of 24,000 and 41,000 *g* respectively are needed. A refrigerated centrifuge developing such forces was not available, and the following modifications were introduced to separate the particles at lower centrifugal forces.

The liver was pressed through a 1 mm. mesh screen; 15–20 g. of the resultant pulp were ground for 5 min. in a mortar and 100 ml. of 0·88 M-sucrose solution added over a period of 10 min. with continuous grinding. The suspension was centrifuged at 600 *g* for 10 min. and the deposit of intact liver cells, debris and nuclei discarded. The supernatant was centrifuged twice more at the same speed, the small sediments being discarded.

The suspension of cytoplasmic constituents, usually divided in two to four tubes, was now centrifuged at 18,000 *g* for 45 min. to throw down the large granules. The supernatant, SII, was set aside. The deposits were pooled with the help of the small amounts of SII left behind and

spun again to separate a little more SII. The residue of large granules was suspended in the sucrose solution and centrifuged at 18,000 *g* for 40 min.; this was repeated three times, the supernatants being discarded. As previously observed by Hogeboom *et al.*, streaming birefringence could be readily demonstrated in the suspensions of large granules in sucrose solutions. After the purification, the preparation under the phase contrast microscope was seen to consist largely of rod-like particles. For chemical analysis, the large granules were rapidly suspended in a saline bicarbonate solution (0·85% (w/v) NaCl containing 0·05M-$NaHCO_3$), centrifuged at 18,000 *g* for 10 min. and the deposit immediately dried from the frozen state.

The density of supernatant SII in four experiments was found to vary between 1·1091 and 1·1098 at 2°. This solution was dialysed under pressure against distilled water for 20–24 hr. The volume of the supernatant usually doubled during the dialysis, the density of the final solution varying between 1·019 and 1·011 at 2°. The dialysed supernatant, SII, was first clarified by centrifugation at 2000 *g* for 15 min., the small deposit discarded, and the clear supernatant from this centrifuged at 18,000 *g* for 2 hr. The sediment, a reddish brown transparent pellet, will be referred to as the small granule fraction. The supernatant from this was centrifuged at 18,000 *g* for 2 hr. and the very small sediment which was usually deposited added to the first sediment. The final supernatant from this centrifugation, SIII, was dried from the frozen state.

The sedimented small granules were resuspended in the saline-bicarbonate solution and centrifuged at 18,000 *g* for 2 hr. This was repeated, and the final deposit dried from the frozen state.

Chemical analysis. The purified granule fractions were analysed for total lipid, phospholipin, nucleic acid P and total N. Whole liver tissue (a dried sample of the liver pulp) was analysed for total lipid and phospholipin, and the supernatant fraction (SIII) for phospholipin.

The dried large and small granule fractions and the whole liver samples were extracted four times by boiling with an ethanol-ether mixture (3:1, v/v) and finally once with ether. All the lipid was considered to be removed by this treatment. The fat-free residues of the large and small granule fractions were dried at 37°; the difference in dry weights before and after extraction was taken as the total lipid content of the sample, and was expressed as a percentage of the dry weight before extraction.

The combined extracts from each sample were evaporated to dryness under reduced pressure (20–40 mm. Hg) with N_2 passing through the extract. The dry residue was extracted three times with light petroleum (b.p. 40–60°). Since the animals were injected with inorganic radioactive phosphate (see Part 2), it was necessary to get rid of traces of contaminating radioactive phosphate from the phospholipins. Ten drops of a saturated aqueous solution of Na_2HPO_4 were therefore added during the light petroleum extraction and the extracts vigorously shaken. It has been shown by Fries, Schachner & Chaikoff (1942) that shaking an ethereal solution of phospholipins with saturated Na_2HPO_4 solution effectively removed traces of radioactive inorganic $PO_4^{\equiv}$ from the phospholipins. The extract was cleared by centrifugation, any particulate matter and inorganic phosphate being discarded in the lower aqueous phase. The clear extract was concentrated to about 2 ml., and the phospholipin precipitated by adding acetone and a saturated solution of ethanolic $MgCl_2$ as described by Bloor (1929). The phospholipins were dissolved in moist ether, and phospholipin P estimated colorimetrically by Allen's (1940) method. Instead of $HClO_4$ as described by Allen, 5N-H_2SO_4 and H_2O_2 ('100 vol.') were used for the digestion. (The micro-analytical reagent grade H_2O_2, supplied by British Drug Houses Ltd., was used throughout this work.) The dried supernatant SIII was similarly analysed.

The P and N contents of the fat-free, dry, liver fractions were determined on 10 mg. samples in duplicate. The N content was determined by a micro-Kjeldahl method; 50% (v/v) H_2SO_4 containing 1% (w/v) SeO_2 and H_2O_2 ('100 vol.') were used for the digestion.

It was assumed that all low molecular weight P compounds were removed in the purification process and therefore the P remaining in the fat-free residue must be nucleic acid P. This P was multiplied by 1·7 to give nucleic acid N and by 9·01 to give the nucleic acid value. The nucleic acid N was subtracted from the total N to give protein N and this was multiplied by 6·25 to give total protein.

RESULTS

In Table 1 are given the values obtained by chemical analyses of whole liver and of the large and small granule fractions from it. The total lipid and more particularly the total phospholipin contents of whole liver are seen to be reasonably constant. In view of this, it is surprising that the total lipid of the large granule fractions is quite variable, which suggests that this fraction is not homogeneous. In appearance the large granule sediment was never homogeneous, two layers being always present. Attempts to separate these by further differential centrifugation, however, were not successful. The total lipid content of the small granules is greater and more constant than that of the large granules. This greater uniformity suggests that the fraction might be homogeneous. This was supported by preliminary electrophoretic experiments in a potassium phosphate buffer, of ionic strength $I = 0{\cdot}2$ and pH = 8·0, in which a single boundary was apparent. The large granule fraction was not similarly examined.

It is suggested here that the centrifugal separation of the large from the small granules may depend mainly on the differences in their respective lipid contents, and hence on the differences in the density of the particles, rather than on the differences in their size.

The nucleic acid values of the large and small granule fractions show the greatest variation, the averages, however, being approximately the same in each. The variation may be due to the method of estimation, depending as it does on the assumption that all low molecular weight phosphorus compounds are removed in the purification process. Estimation of the ribonucleic acid content of these fractions by colorimetric methods based on ribose was unsatisfactory because of other interfering compounds, e.g. glucose from glycogen breakdown.

Table 1. *Lipid, nucleic acid and protein content of whole liver and of large and small granules obtained from it*

(All results are expressed as percentage of dry weight. L. = liver; L.G. = large granules; S.G. = small granules. 'Total' (last two columns) = sum of total lipid, nucleic acid and protein.)

Exp. no.	Total lipid			Phospholipin			Nucleic acid		Protein		Total	
	L.	L.G.	S.G.	L.	L.G.	S.G.	L.G.	S.G.	L.G.	S.G.	L.G.	S.G.
20	15·8	14·1	42·3	12·6	13·1	31·0	—	3·33	—	45·6	—	91·2
21	16·5	30·6	41·9	13·5	17·5	31·8	2·34	3·00	63·8	50·5	96·7	95·4
22	15·6	27·5	45·2	13·6	18·5	34·0	2·95	2·38	62·0	49·4	92·5	97·0
23	21·4	36·0	44·7	12·0	13·7	26·2	3·92	3·24	49·0	51·0	88·9	99·0
26	—	29·6	44·2	—	19·6	35·7	2·45	2·9	61·6	50·8	93·7	97·9
27	—	28·0	41·6	—	19·0	34·3	2·45	2·81	64·3	54·0	94·8	98·5
29	17·5	41·0	45·0	—	17·8	—	3·0	2·6	49·6	49·7	94·6	97·3
30	18·2	37·2	43·0	14·0	17·2	—	3·4	1·25	52·3	53·8	92·8	98·1
32	18·3	30·9	42·6	13·6	17·8	30·2	2·5	2·64	61·5	43·2	94·9	88·4
33	18·8	25·6	40·1	13·1	19·4	29·6	2·75	1·74	64·4	—	92·8	—
34	18·7	28·2	43·7	14·8	17·6	28·2	1·8	2·9	65·3	55·1	95·3	101·7
35	19·4	26·3	46·2	15·3	18·5	—	2·1	2·0	67·5	57·0	95·4	99·2
Mean	**18·0**	**29·6**	**43·4**	**13·6**	**17·5**	**31·2**	**2·7**	**2·6**	**60·2**	**50·4**	**94·0**	**96·6**
S.D.	1·76	6·82	1·77	1·02	2·14	2·97	0·57	0·62	6·3	3·5	2·1	3·8

Table 2. *Comparison of the analytical data obtained for the large and small granule fractions of rabbit liver with those reported by other investigators*

	Large granules					Small granules					
	Total lipid	Phospholipin	Total N	Total P	N.A.P.* (μg.)/total N (mg.)	Total lipid	Phospholipin	Total N	Total P	N.A.P.* (μg.)/total N (mg.)	Animal species
	(% dry wt.)					(% dry wt.)					
Claude (1946*b*)	25	16	10–12	0·9–1·3	—	40–45	29	9·15	1·51	—	Pig
								8·95	1·74	—	Rat
Hogeboom *et al.* (1948)	—	—	—	—	15·5	—	—	—	—	58	Rat
Present author	29·6	17·5	10·5	1	70	43·4	31·2	9	1·5	80	Rabbit

(N.A.P. = Nucleic acid phosphorus.)

The protein values also show considerable variation, particularly those for the large granule fraction. In the latter case, however, the variation might be due to the inhomogeneity previously mentioned. No correlation was found between the amounts of the various components in each fraction. The total lipid, nucleic acid and protein account for 94 % of the total solids of the large granules and for 96·6 % of the small granules. Thus, these fractions contain small amounts of other substances. Claude (1946*b*), for example, found approximately 0·5 % of inositol in the large granules. No attempt has been made in the present work to identify the unestimated components. In Table 2 the results of this investigation are compared with those of Claude (1946*b*) and Hogeboom *et al.* (1948). The values for rabbits are in good agreement with those obtained for rats and guinea pigs. The unequal distribution of nucleic acid between the large and small granules in rat livers (Hogeboom *et al.*) has not been found with rabbit liver.

Distribution of phospholipin, nucleic acid and protein between large and small granule fractions

The yield (dry weight/dry weight) of fractions from liver pulp was as follows: large granules, 3·5 ± 0·9 %; small granules, 4·9 ± 0·8 %; the ratio of the two being 0·73 ± 0·11. If it is assumed that this is also the ratio of the granules in the liver-cell cytoplasm, and if the small fractions discarded during the fractionation are neglected, then the

Table 3. *Distribution of constituents between large and small granules of rabbit liver*

	Large granules (%)	Small granules (%)
Nucleic acid	43	57
Protein	46	54
Total lipid	33	67
Phospholipin	29	71

distribution of the cytoplasmic constituents between the two types of particles may be calculated as shown in Table 3. These values must be very approximate because of the great technical difficulties of the fractionation procedure. The distribution of phospholipin, among the large granules (1), small granules (2) and supernatant (3), was similarly calculated to be as follows: (1) 26·4, (2) 64·6, (3) 9% respectively.

2. INVESTIGATIONS USING RADIOACTIVE PHOSPHORUS

The object of these experiments was to find out if there is a site in the liver cell where most of the phospholipin is synthesized. Neutral sodium phosphate containing ^{32}P was injected into rabbits and the various liver fractions prepared as described in Part 1. The specific activities (counts/min./mg.) of phospholipin phosphorus of the fractions were determined, and expressed as percentage of ^{32}P injected/kg. of body weight (counts/min./kg.).

METHODS

Administration of ^{32}P. In a few experiments of 4 hr. duration, the ^{32}P (as Na_2HPO_4 in saline) was administered to the rabbits either as a continuous intravenous infusion, or in subcutaneous injections repeated at 20 min. intervals in order to maintain a constant level of inorganic ^{32}P in the plasma, as described by Hevesy & Hahn (1940). Under these conditions, the specific activities of all P compounds—including those of the liver phospholipin—should rise until they reach the level of the specific activity of the plasma inorganic P.

In the majority of experiments, however, inorganic phosphate labelled with ^{32}P was given in a single subcutaneous injection, and the animals killed 2–72 hr. later. The total dose of ^{32}P administered was usually 200 μc./rabbit.

Analysis of blood. Blood was obtained from the rabbits as described in Part 1. It was collected in a vessel containing heparin, cooled immediately, and the plasma drawn off after centrifugation at 2°. Approximately 15 ml. of plasma were extracted with 20 vol. of ethanol-ether (3:1, v/v) and the phospholipins obtained as described previously. Approximately 5 ml. of plasma were mixed with an equal volume of 10% (w/v) trichloroacetic acid, the precipitated protein centrifuged off and the inorganic phosphate precipitated as $MgNH_4PO_4$.

Analysis of liver. A sample of liver was fractionated by differential centrifugation as described in the previous section. Liver-cell nuclei were prepared by the method of Mirsky & Pollister (1946) and dried from the frozen state. The phospholipins of the fractions were extracted and purified as already described, and were digested with 5N-H_2SO_4 and H_2O_2 ('100 vol.'). The inorganic phosphate thus obtained was precipitated as $MgNH_4PO_4$.

Immediately after removal of the liver, a small portion was blotted quickly with filter paper and dropped into a freezing mixture of acetone and solid CO_2. The frozen tissue was powdered in a steel mortar previously cooled with the freezing mixture. The crushed liver (about 5 g.) was shaken by hand with cold 5% (w/v) trichloroacetic acid (10 ml.) and the mixture filtered. Inorganic phosphate was separated from the extract as $MgNH_4PO_4$.

Assay of ^{32}P. All P samples were obtained as dry $MgNH_4PO_4$. In early experiments samples containing 50–300 μg. of P were dissolved in 0·5 ml. 0·1N-HCl. Of this, 0·3 ml. was pipetted accurately on to a small nickel disk, whilst 0·1 ml. was used for the determination of P. The deposits on the disks were dried by gentle heating, and the radioactivity was measured with a bell-shaped Geiger-Müller counter.

Owing to the low radioactivity of some of the samples, and the difficulty of obtaining a uniform layer containing more than 300 μg. of P on the disk, a cylindrical, jacketed counter, described by Veall (1948) and suitable for the measurement of radioactivity of solutions, was later used. Amounts up to 5 mg. of $MgNH_4PO_4$ were dissolved in 1 ml. N-HCl and 10 ml. of water added; the radioactivity of 10 ml. of the mixed solution was then measured and samples of the solution were used for P estimations (in duplicate).

At least 5000 and usually 10,000 counts from each specimen were taken, giving a statistical accuracy of at least $\pm 1{\cdot}5\%$. The specific activity determinations are accurate to $\pm 3\%$.

RESULTS

Table 4 contains the results of an experiment in which ^{32}P was injected subcutaneously every 20 min. over a period of 4 hr. Samples of blood (5 ml.) were taken at intervals after the first injection as indicated in Table 4, and the plasma inorganic phosphorus isolated as previously described. The final blood sample was taken and the liver removed 20 min. after the last injection.

Table 4. *Specific activity of plasma inorganic* P *and of liver*-P *fractions after subcutaneous injection of* ^{32}P

(^{32}P injected every 20 min. over a period of 4 hr.)

A. Change in plasma inorganic P with time

Time after first injection (min.)	Plasma inorganic P specific activity (counts/min./mg. P)
55	77
95	77·5
135	77
175	97
215	77
235	70

B. Specific activity of liver and plasma-P fractions 235 min. after first injection

	Specific activity (counts/min./mg. P)
Liver	
Inorganic P	39
Phospholipin P, small granules	1·30
Phospholipin P, large granules	1·14
Phospholipin P, nucleus	1·00
Phospholipin P, supernatant	0·73
Plasma	
Inorganic P	70
Phospholipin P	0·83

The specific activities of the various phosphorus fractions shown are typical. The specific activity of the inorganic phosphorus in the plasma is maintained

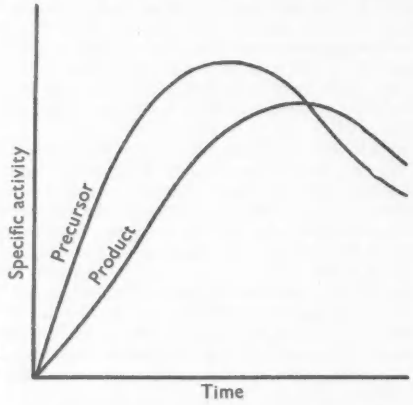

Fig. 1. Theoretical relationship between the specific radioactivity of precursor and its product.

at a reasonably constant level. Over the 4 hr. period, the specific activity of the liver inorganic phosphorus (not corrected for extracellular inorganic phosphate) rises to about half that of the plasma inorganic value, but the uptake of ^{32}P into the phospholipin fractions is much slower. The two main cytoplasmic fractions, small and large granules, are similar to each other in value, with the former always slightly higher. The specific activities of the phospholipins of the nucleus and of the supernatant are somewhat lower, the value of the latter being lower than that of the plasma phospholipin.

The result indicates that phospholipin fractions of different specific activities may exist in the liver cell under the conditions of this experiment. However, this experiment does not indicate whether the phospholipins in the fractions are synthesized independently of one another in the cell, or whether they are synthesized in one fraction—viz. the small granules, as this fraction has the highest specific activity—and are transferred from this fraction to the others.

Zilversmit, Entenman & Fishler (1943) have established mathematically the relationship between the specific activity of a labelled precursor and that of its product in experiments in which the specific activity of the precursor is a function of time. In such experiments it is necessary to give only one injection of the labelling agent (e.g. ^{32}P). This relationship is illustrated graphically in Fig. 1, from which it can be seen that the specific activity of the product at its maximum is equal to the specific activity of its precursor at the same time. Before this maximum has been reached the specific activity of the compound must be less, and afterwards it must be greater, than the specific activity of the precursor. These criteria have been employed in our experiments in which sixteen rabbits were killed at intervals of 2–72 hr. after an injection of radioactive phosphate.

Since it is necessary to kill the rabbit in order to get sufficient liver for analysis, each animal can provide data for one point only on the time curve of liver phosphorus turnover. The metabolic idiosyncrasies of rabbits are such that it is impossible to predict beforehand how much labelled phosphate to inject in order to create exactly the same level of ^{32}P in any plasma or tissue fraction in all animals at the same time. To circumvent this difficulty a

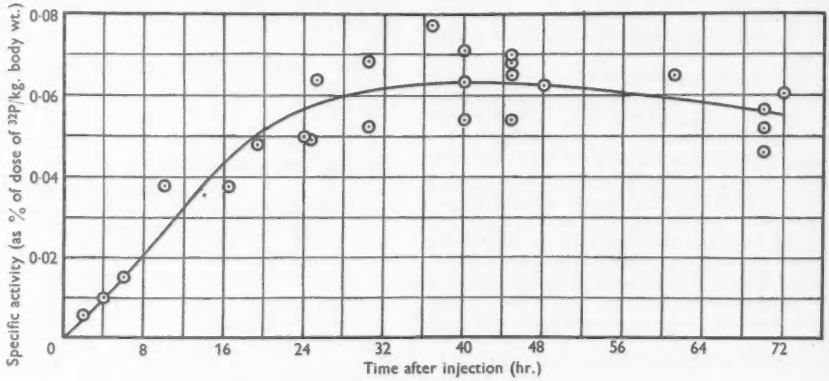

Fig. 2. Change in specific radioactivity of plasma phospholipins of rabbits injected with a single dose of inorganic ^{32}P.

specific activity-time curve of plasma phospholipins was established for an independent series of rabbits which were bled at four different intervals from 2 to 72 hr. after an injection of labelled phosphate.

This curve, shown in Fig. 2 and reproduced again in Fig. 3, was taken as the reference standard and all specific activities of the plasma-phospholipin phosphorus from the main series of experiments were adjusted to fall on this curve. Correspondingly, the specific activities of the three liver phospholipin fractions were multiplied by the same factor. Thus this correction does not alter the ratios of the specific activities of the three liver phospholipin fractions to that of the plasma phospholipins; only the absolute values are affected.

From Fig. 3 it can be seen that the specific activity-time curves of the three liver fractions remain independent of each other during the experiment. That is, not one of these components behaves as a precursor with respect to any of the others, according to the conditions set down by Zilversmit *et al.* (1943).

The first set of experiments showed that phospholipin fractions of three different specific activities existed in the liver cytoplasm, and the question arose whether the phospholipins in the fractions were synthesized separately, or whether the phospholipins in one fraction, viz. the small granules, might be the precursor of the phospholipins of the others. In applying the criteria laid down by Zilversmit *et al.* (1943) for the establishment of a precursor, several assumptions, as recognized by these authors, are made. These are: (1) a steady state, in which the amount of compound present in the tissue studied must be constant during the interval over which the observations are made; (2) the rate of synthesis and degradation of the compound must be constant during the experiment; (3) the appearance and disappearance of all molecules must proceed at random, i.e. the specific activity of portions of the compound breaking down (or leaving the tissues) is equal to the specific activity of the total amount of the compound present in that tissue. In addition to these, a fourth assumption is involved in the present investigation of the components obtained by fractionation. Since they are not simple compounds, it must be shown that their properties are reproducible from experiment to experiment. The results in Table 1 show that, as far as chemical composition is concerned, this assumption may be justified.

As fully matured rabbits of 2·3–2·8 kg. body weight were chosen, the first and second assumptions

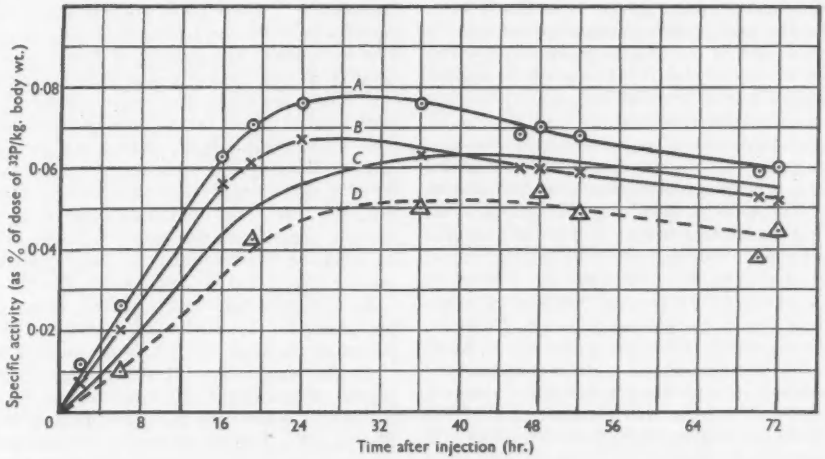

Fig. 3. Change in specific radioactivity of liver and plasma phospholipins of rabbit injected with a single dose of inorganic ^{32}P. *A*, Phospholipins extracted from small granules; *B*, phospholipins extracted from large granules; *C*, plasma phospholipins; *D*, phospholipins extracted from liver 'supernatant' after large and small granules have been removed.

DISCUSSION

Hevesy's (1945) original observation that the rate of renewal of phospholipin phosphorus in the liver nuclei is less than the rate in cytoplasm has been confirmed in the present series of experiments, though there does exist in the cytoplasm a small component (the supernatant) with an even slower rate of renewal.

are justified. In the present investigation, the third assumption is perhaps not justified, as there is no evidence that the phosphorus of the phospholipin molecules distributed throughout a small or large granule is of uniform specific activity at any moment. If the latter assumption is made however, the curves in Fig. 3 indicate that phospholipin synthesis (or at least the step involving the incorporation of phosphorus into the phospholipin molecule) may take place in at least three sites in the liver cytoplasm. [It will be noticed from the curves in Fig. 3, however, that the large granule phospholipins appear to be the precursor of the plasma phospholipins.] The criteria of Zilversmit *et al.* (1943) can only be applied in this case if it is known that the plasma phospholipins are entirely derived from the liver, i.e. that no phospholipin enters the plasma from any other source to alter significantly the specific activity of the total plasma phospholipins. As previously mentioned, the work of Fishler *et al.* (1943) and Entenman *et al.* (1946) has demonstrated that liver is the principal tissue in the body concerned with the production and removal of plasma phospholipins. More recently, Artom & Swanson (1948) have shown that phospholipins may reach the plasma from the intestine, though it appears that they do so only in very small amounts. If the assumption is made that the liver is the sole source of plasma phospholipins, then the curves in Fig. 3 indicate that plasma phospholipins are derived only from the large granule fraction. On the basis of this assumption, it can be calculated from the distribution figures previously presented that, under the conditions of these experiments, only 25–30 % of the total phospholipin formed in the liver is transferred to the plasma. Most of the phospholipins formed in the liver remain inside the cell, and presumably are used in the normal metabolism of the cell.

It has been suggested by Claude (1943) that the large granule fraction and the microsome or small granule fraction have a common origin, or that one type of granule may contribute to the constitution of the other. Claude quotes observations which appear to support the latter possibility. While the results of the present investigation do not directly confirm or deny these possibilities, it is significant that, as far as their phospholipin components are concerned, the metabolism of the two fractions is different, and it is therefore unlikely that one fraction is derived from the other.

SUMMARY

1. A modification of the method of Hogeboom *et al.* (1948) for the fractionation of liver cytoplasm by differential centrifugation has been used for the preparation of three fractions, large granules, small granules (microsomes) and supernatant, from rabbit-liver cytoplasm. The method of Mirsky & Pollister (1946) has been used to prepare rabbit-liver nuclei.

2. The microsome and large granule fractions from twelve rabbit livers have been analysed for total lipid, phospholipin, protein and nucleic acid content. Total lipid, protein and nucleic acid account for 94 % of the large granules and 96·6 % of the small granules. The total lipid (including phospholipin) content constitutes the main chemical difference between the two fractions, the large granules containing 29·6 % and the microsomes 43·4 %.

3. Approximately 26·4 % of the liver cytoplasmic phospholipins was in the large granules, 64·6 % in the small granules, and 9 % in the supernatant.

4. The metabolism of the phospholipin phosphorus of the cytoplasmic fractions and the nucleus has been studied with the aid of radioactive phosphorus (^{32}P). It is shown that at any time after the injection of radioactive phosphate into rabbits there exist in the liver cytoplasm at least three phospholipin fractions with different specific activities. The original observation of Hevesy (1945), that the rate of renewal of phospholipin phosphorus in the liver nuclei is slower than in the cytoplasm, has been confirmed.

5. The results suggest that phospholipins are being synthesized separately and metabolized independently of each other in different morphological structures of the liver cell.

I wish to thank Dr A. S. McFarlane for his stimulating interest in this work and for valuable discussions during its progress. I am indebted to Dr G. Popják for carrying out all the operative procedures.

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Isolation of L-Threonine from Proteins

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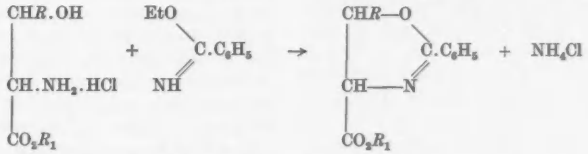
L-Threonine was first isolated from fibrin by McCoy, Meyer & Rose (1935), after a series of careful investigations extending over a number of years. It was shown to be an essential growth factor for rats. In spite of the obvious biological importance of threonine, its metabolism in animals has not been studied by the modern isotope techniques which have yielded such valuable results in the amino-acid field. This is probably due to the difficulties involved in its isolation in a pure state. McCoy *et al.* (1935) worked on a very large scale and obtained only a minute yield of pure substance. Subsequently, Woolley & Peterson (1937) isolated threonine from an autolysate of the mycelium of *Aspergillus sydowi* using a different procedure. The yield of threonine was considerably higher, but in this case also the work was carried out on a large quantity of starting material. Sharp (1939) isolated a sample of threonine from rabbit myosin by fractional distillation of the esters of the 'monoamino-acid fraction', but oxidation with periodate revealed that the preparation was not pure (Martin & Synge, 1941). The latter authors were able to isolate a hydroxyamino-acid fraction from wool hydrolysate by their acetylation-benzoylation procedure. Pure serine was isolated from this fraction in fairly good yield, but the isolation of threonine was not attempted. Freudenberg, Walch & Molter (1942) isolated threonine from human blood-group A substance. This publication has been seen only in abstract form without details.

For adequate isotope analyses and determinations of purity about 25 mg. of pure threonine would be required. Owing to the small amount of threonine generally present, at least 0·5 g. of protein would be required to obtain the above quantity. Since the substance sought was a minor constituent of a complex mixture considerable loss seemed inevitable; to allow for this experiments were conducted with 5 g. of protein. Some large-scale experiments were also carried out. The method of isolation which has been worked out makes use of certain novel properties of the hydroxyamino-acids which have been briefly described previously (Elliott, 1948) and are now discussed in detail.

Separation of serine and threonine from protein hydrolysates by oxazoline derivatives

It has been shown by Elliott (1948, 1949) that serine and threonine can be converted into cyclic oxazoline derivatives by reaction of the ester hydrochloride with benziminoethyl ether in the cold. Under



these conditions amino-acid esters lacking the β-hydroxyl group also react to give *N*-substituted imino ethers (Cornforth & Cornforth, 1947):

$$C_6H_5.C(OEt):NH + HCl.NH_2.CHR.CO_2Et \rightarrow C_6H_5.C(OEt):N.CHR.CO_2Et + NH_4Cl.$$

When this reaction was applied to the mixture of ester hydrochlorides resulting from the esterification of a protein hydrolysate a complex mixture resulted; this contained the oxazolines derived from serine and threonine and the substituted imino ethers from the remaining amino-acids.

In this work both ethyl and *iso*propyl alcohols have been used for esterifying the protein hydrolysates. Synthetic studies (Elliott, 1949) have shown that the *iso*propyl esters give somewhat higher yields in the reaction with benziminoethyl ether.

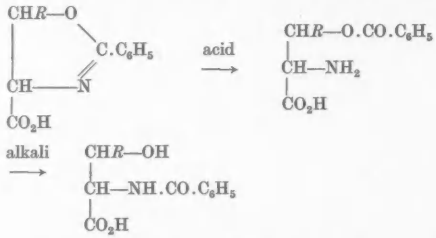
By means of a rough fractional distillation at low pressure considerable enrichment of the oxazoline fraction was effected without loss, the main contaminants being the imino ethers derived from glycine, alanine and valine. In contrast to amino-acid esters, both classes of derivatives obtained by the reaction with benziminoethyl ether are quite stable to distillation and can be kept in closed vessels for several weeks without appreciable decomposition. At an early stage in the investigation it was realized that the separation of the oxazolines in a state of purity by fractional distillation would be impossible except in a fractionating column with a high theoretical plate number. Alternative methods of

purification were sought. It was shown by Abraham, Baker, Chain, Cornforth, Cornforth & Robinson (1945) that a substituted imino ether of the type formulated above was hydrolysed by alkali to the corresponding acylamino-acid:

$$C_5H_{11}.C(OMe):N.CH_2.CO_2Et \rightarrow C_5H_{11}.CO.NH.CH_2.CO_2H.$$

Some preliminary experiments with the compound $C_6H_5.C(OEt):N.CH_2.CO_2Et$, obtained from glycine ethyl ester hydrochloride and benziminoethyl ether, showed that the above hydrolysis was almost quantitative under suitable conditions. Although oxazolines can also be considered as substituted imino ethers it was expected that they would be more stable to alkali by virtue of the cyclic system. Studies with synthetic compounds showed that this was the case; when the oxazolines derived from serine or threonine were boiled with dilute aqueous alkali the ring system remained intact and only the carbethoxyl group was hydrolysed. This alkali treatment, when applied to the oxazoline fraction from a protein hydrolysate, was expected to produce in solution the sodium salts of the oxazoline carboxylic acids, together with the sodium salts of hippuric acid, benzoylalanine, etc. This was the case only when hydrolysis was carried out as rapidly as possible using relatively strong aqueous-ethanolic alkali; milder conditions of hydrolysis led to almost complete destruction of the threonine. The reason for this is not clear, but it is probable that under alkaline conditions the esters condense with one another, the process being immediately arrested once the carbethoxyl groups have been hydrolysed.

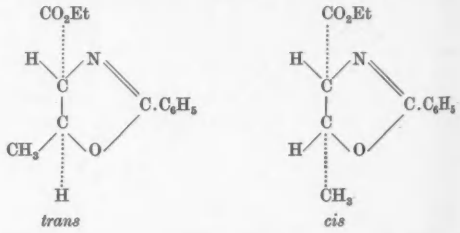
After hydrolysis of the oxazoline fraction, the solution was acidified with hydrochloric acid to pH 1. This caused an immediate precipitation of an oily mixture of hippuric acid, etc., but the oxazoline carboxylic acids remained in solution as the hydrochlorides. Synthetic studies had established that the oxazoline ring was rapidly opened at pH 1 to give the corresponding *O*-benzoyl derivative (Elliott, 1949).



The acidified solution described above was then thoroughly extracted with ethyl acetate and the organic extract rejected. The aqueous layer contained only the *O*-benzoyl derivatives of serine and threonine as their hydrochlorides; when this solution was made alkaline to phenolphthalein the benzoyl group moved instantaneously on to the nitrogen atom. A mixture of benzoylserine and benzoylthreonine free from impurities was then obtained by a second acidification and solvent extraction. The above series of transformations can be carried out quite easily in a few hours. The remaining problem was the separation of serine from threonine, but before discussing this it is necessary to consider the stereochemical aspects of this problem.

Separation of L-*benzoylthreonine from* DL-*benzoylserine*

As a result of experiments, which will be described later, convincing proof has been found that benziminoethyl ether reacts with serine and threonine ester hydrochlorides to produce oxazolines with complete retention of configuration at the α- (and in the case of threonine also at the β-) carbon atom. It has been shown (Elliott, 1948, 1949) that oxazolines of the type under discussion most probably possess an active α-hydrogen which is mobilized by the action of hot alkali. The discovery that the serine, isolated from the serine-threonine mixture as described below, was completely racemized was therefore in accordance with expectations. The configuration of the threonine derivative, however, appeared to be completely preserved. The reason for this becomes obvious when the following facts are considered. Racemization at the α-carbon atom in the threonine derivative would produce not the DL-oxazoline, but a mixture of oxazolines derived from L-threonine and D-*allo*threonine. These can be assigned *trans* and *cis* structures respectively, on the basis of the configurational relationship between L-threonine and D-threose established by Meyer & Rose (1936). These two forms must be in equilibrium in highly alkaline



solution, because of the mobile α-hydrogen atom, but it has been shown (Elliott, 1948, 1949) that the equilibrium favours the *trans* form to such an extent that the *cis* form cannot be isolated.

The separation of DL-benzoylserine from L-benzoylthreonine was carried out in two ways. In the first method the mixture of benzoyl compounds was hydrolysed and the amino-acids separated from one another by partition chromatography on a column of potato starch. The use of potato starch in partition

chromatography was first described by Synge (1944). Although development of the chromatogram required several days, very satisfactory results were obtained on a small scale. Larger quantities of material were more easily handled by countercurrent distribution after a preliminary step in which most of the benzoylserine was removed as the sparingly soluble β-phenylethylamine salt. In contrast to DL-benzoylthreonine, which also forms a sparingly soluble β-phenylethylamine salt (Carter & Risser, 1941), L-benzoylthreonine forms a very soluble salt which is not crystalline. When a high yield of pure material is desirable in the separation of two substances it is advantageous to use the principle derived by Bush & Densen (1948) in which $V_x/V_y=\sqrt{(1/K_aK_b)}$, where V_x and V_y are the volumes of solvent used in each equilibration, and K_a, K_b are the partition coefficients of the two substances to be separated. It is also advantageous to carry the procedure through a 'diagonal stage' (see Bush & Denson, 1948, p. 123). The distribution curves of the two substances are then mirror images of each other and, providing K_a/K_b is sufficiently large, the major part of one solute will accumulate in one solvent, while the second solute will accumulate in the other solvent. The upper and lower layers can then be separately pooled and the pure components isolated from them.

In initial experiments ether and water were found to give the largest ratio K_a/K_b. The ratio of solvents to be used in each equilibration was then calculated by applying the above formula as follows: K_a (benzoylthreonine) = 0·218; K_b (benzoylserine) = 0·100; V_x = volume of ether; V_y = volume of water; $V_x/V_y=\sqrt{(1/0{\cdot}0218)}=1/0{\cdot}148$. Let p_a = the fraction of benzoylthreonine which remains in the upper phase at each equilibration. Then

$$p_a/1-p_a=K_aV_x/V_y,$$
$$p_a=K_aV_x/(K_aV_x+V_y)=0{\cdot}218/(0{\cdot}218+0{\cdot}148)$$
$$=0{\cdot}60.$$

In the paper by Bush & Densen (1948), already referred to, a series of curves are given from which the fraction of solute in the pooled upper or lower layers can be found for all values of p and values of n (number of equilibrations) up to 100. Reference to these curves showed that a twelve-funnel process should yield 80% of the original amount of benzoylthreonine from the pooled ether layers. If the bulk of the benzoylserine were removed previously as the β-phenylethylamine salt this benzoylthreonine should be more than 80% pure. It was found that recrystallization from water removed the remaining impurities, but losses were considerable.

The advantage in using buffer solutions, particularly concentrated ones, in countercurrent distribution methods has been pointed out by Craig, Golumbic, Mighton & Titus (1945). After a series of experiments it was found that ethyl acetate and a 2M-phosphate-citrate buffer at pH 3·6 gave the considerably increased value of 2·9 for K_a/K_b. In this case, by calculation as above, a ten-funnel process should raise the purity of the benzoylthreonine to 96%, and by repetition of this process on the purified material the purity of the benzoylthreonine should be raised almost to 100% with a loss of only 20%. Citric acid is not completely insoluble in ethyl acetate, and it was found preferable to remove this by a third countercurrent distribution of the benzoylthreonine between ethyl acetate and water. In this case K_a/K_b ($K_b=K$ for citric acid) was very high (39·5), so that the removal of citric acid was a simple matter. In practice it was found that the degree of purification was somewhat less than that calculated and the final product had a purity of 96·3%. This figure was obtained by periodate analysis of the L-threonine resulting from acid hydrolysis of the benzoyl derivative. The amino-acid had the correct specific rotation, but this did not exclude the possibility that a very small amount of D-*allo*threonine was present.

Fortunately, for the purpose for which this method was worked out, the optical purity of the threonine was of no consequence; it was necessary only to ensure that the product was free from other amino-acids, particularly serine. Although the available evidence suggests that the threonine isolated was optically pure, this must await confirmation by a more sensitive method.

Isolation of serine from the oxazoline fraction without racemization

In order to prove that no racemization occurred when benziminoethyl ether reacted with serine and threonine ester hydrochlorides it was necessary to isolate L-serine from the oxazoline fraction by some method which avoided treatment with alkali at any stage. It has already been stated that the oxazoline ring in the compounds under discussion is readily opened in aqueous solution at pH 1. At pH 4, however, no ring opening could be detected, at least for short periods of time. Model experiments with the imino ether derived from glycine, on the other hand, showed that it was rapidly hydrolysed at pH 4 into ethyl benzoate and glycine ester. This is exactly analogous to the fission of the oxazoline ring to give the corresponding *O*-benzoyl compound:

$$C_6H_5.C(OEt)\vdots\!=\!N.CH_2.CO_2Et$$
$$\rightarrow C_6H_5.CO_2Et+NH_2.CH_2.CO_2Et.$$

Oxazolines, being weak bases, should be extracted by ether from a buffer at pH 4, whereas the strongly basic amino-acid esters should remain in the aqueous phase.

To test out these ideas experiments were conducted on silk sericin, which contains a very high

proportion of hydroxyamino-acids (Nicolet & Seidel, 1941). This protein was obtained in solution, in partly degraded form, by a very short treatment of raw silk with hot dilute acid, the fibroin being practically unaffected. The oxazoline fraction from silk sericin was shaken with an acetate buffer at pH 4 for 3 hr., the insoluble oil extracted into ether, the combined extracts evaporated and the residue hydrolysed. Analyses showed that the hydroxyamino-acid nitrogen content of the solution, determined by periodate ammonia estimation, was only slightly less than the amino nitrogen, indicating almost complete purification of the hydroxyamino-acid fraction. L-Serine was isolated from the hydrolysate as the *p*-hydroxyazobenzene-*p'*-sulphonic acid salt by the method of Stein, Moore, Stamm, Chou & Bergmann (1942) and had the correct specific rotation. Unfortunately, this alternative method, which seemed promising for the isolation of threonine as well as serine, gave unsatisfactory results with a casein hydrolysate and cannot be of general application.

EXPERIMENTAL

Methods of analysis. Serine and threonine were determined by periodate oxidation according to the method of Rees (1946). Periodate NH_3 was determined by the method of Van Slyke, Hiller & MacFadyen (1941). The same apparatus was used for both these determinations. Amino-N determinations were carried out in the Van Slyke gasometric micro-apparatus. Partition coefficients were measured by equilibrating the substance between equal volumes of the two solvents and determining the amount in each layer by titration, or by Kjeldahl determinations when buffer solutions were used.

Hydrolysis of the proteins. A good brand of commercial casein was used for most experiments. A preparation of mixed rabbit-muscle proteins was also used. These were hydrolysed by refluxing 16–24 hr. with 5 parts (w/v) of 6N-HCl and were filtered from acid-insoluble humin. A preparation of raw silk, known commercially as 'Turkish Knubbs', was stirred with 20 parts (w/v) of boiling N-HCl for a minute or so until the sericin had been removed. This was easily judged by microscopic examination of the fibres. The fibroin was then squeezed out, steeped in distilled water and squeezed out again. The two solutions were combined. Hydrolysis of the protein was accomplished by evaporating the solution at ordinary pressure until the acid in the distillate was about 5N. The solution was then refluxed for 4 hr. Amino-N determinations revealed that hydrolysis was complete in this time. In this brand of raw silk, the sericin content, as determined roughly by the weight loss on acid treatment, was 15–20%.

Preliminary treatment of the protein hydrolysates. Silk sericin hydrolysate required no preliminary treatment. Hydrolysates from about 5 g. of casein were diluted with about 5 parts of water, heated to 60° and a small amount of a concentrated solution of phosphotungstic acid stirred in. This removed the acid-soluble humin as a precipitate which settled rapidly. Sufficient phosphotungstic acid was added to render the supernatant liquid almost colourless, but with a comparatively pure protein such as casein it was not necessary to precipitate more than a small amount of the basic amino-acids. When crude proteins were used it was considered advisable to precipitate the basic amino-acids almost completely, as described below. After cooling, the solution was filtered through a Ford Sterilmat F.C.B. grade, extracted twice with a mixture of amyl alcohol 20 parts, ether 25 parts, ethanol 1 part (see Chibnall, Rees & Williams, 1943) and evaporated to dryness under reduced pressure.

On a larger scale it was preferable to remove as much as possible of the neutral, acidic and basic amino-acids. The following is an example of the method used. The hydrolysate from 300 g. of casein was evaporated under reduced pressure to about 540 ml. The solution was saturated with HCl at 0° and allowed to stand 4 days at 0°. To this solution was then added, with stirring, 900 ml. ethanol which had previously been cooled to about −30°; the purpose of this cooling was to prevent a rise in temperature above 0° when the ethanol and the acid solution were mixed. The glutamic acid hydrochloride was filtered through a sintered glass funnel and washed with 300 ml. of ice-cold ethanol. A yield of 67 g. of the hydrochloride was obtained, representing about 80% of the amount present in the protein. It should be emphasized here that quantitative removal of the various fractions was not necessary. The filtrate and washings from the glutamic acid were evaporated to dryness under reduced pressure, redissolved in water and evaporated again. The residue was dissolved in 5·25 l. of N-HCl, the solution heated to boiling and phosphotungstic acid (920 g. dissolved in 1·8 l. water) added fairly rapidly with stirring. After standing at room temperature for 2 days the crystalline precipitate was filtered at the pump, stirred with 750 ml. of 2·5% (w/v) phosphotungstic acid in N-HCl, filtered again, washed with 375 ml. of the same solution on the funnel and finally sucked as dry as possible on the pump. The combined filtrate and washings were saturated with ether, extracted three times with the amyl alcohol-ether-ethanol mixture using 2250 ml. in all and then evaporated to dryness under reduced pressure. This method of precipitating the basic amino-acids is a simplification of the method of Chibnall *et al.* (1943). The residue of amino-acid hydrochlorides was then dissolved in water (750 ml.), the solution cooled and 5N-NaOH added dropwise with stirring, the temperature being kept below 15°, until the pH of the solution was about 6. Crystallization began almost immediately, and after keeping overnight at 0° the crop was filtered and washed with a small quantity of saturated NaCl solution. The combined filtrates were evaporated to obtain a second crop, and so on until the crop of solid consisted almost entirely of NaCl. This procedure served to remove most of the leucine and tyrosine and probably some phenylalanine and methionine. The solution was then strongly acidified with conc. HCl and evaporated to dryness under diminished pressure. This was best carried out in a large flask because of bumping. The residue was extracted thoroughly with boiling ethanol, the NaCl filtered and thoroughly washed with boiling ethanol. The ethanolic extract was then evaporated to dryness under reduced pressure. Analyses showed that about 20% of the threonine was lost during the above operations.

Esterification of the protein hydrolysates

(*a*) *Ethyl esters.* The syrupy residue of amino-acid hydrochlorides was finally dried by evaporation twice under reduced pressure with dry ethanol. The amount of ethanol used for esterification was not critical and 5–20 parts (based

on the weight of protein) were used, the amount depending on the scale. On the small scale it was more convenient to use dilute solutions. A rapid stream of dry HCl was passed through a dry ethanolic solution of the amino-acid hydrochlorides until the solution boiled. It was then cooled and resaturated with HCl. After standing overnight the solution was evaporated to dryness under reduced pressure. To ensure complete esterification the process was repeated, but this may not have been necessary.

(*b*) iso*Propyl esters*. The residue of amino-acid hydrochlorides was dried as before by evaporating with ethanol and then evaporated once with *iso*propanol. The residue was suspended in 10–20 parts of dry *iso*propanol and a rapid stream of dry HCl passed in until the solution boiled. A steady stream of dry HCl was then passed through the solution for 1 hr. and boiling was maintained by application of heat at such a rate that about one-third of the alcohol was allowed to escape through a 15 in. air condenser fitted to the flask. After about 30 min. fresh *iso*propanol was added to compensate for the loss through the condenser. The hydrochlorides slowly dissolved. The solution was then evaporated to dryness under reduced pressure. In the case of both (*a*) and (*b*) the esters were finally dried in a vacuum desiccator over NaOH and H_2SO_4.

Preparation of benziminoethyl ether. Dry HCl was passed into dry ethanol (46 g.) at 0° until 36·5 g. had been absorbed. The solution was then added fairly rapidly to freshly distilled benzonitrile (103 g.) which had also been cooled to 0°. The mixture was allowed to stand 4 days at 0° with careful exclusion of moisture. The solid mass of imino ether hydrochloride was broken up under dry ether, filtered and washed thoroughly with dry ether. A small amount of hydrochloride separated from the ether washings on standing at 0°. The total yield was 141 g. (76%); m.p. 132–134° (decomp.). The hydrochloride (173 g.) was added to a sludge of K_2CO_3 (129 g.), and water (90 ml.) which was covered with a layer of ether and cooled to −5°. After thorough shaking the ether layer was decanted and the sludge washed twice more with fresh ether. The combined extracts were dried over anhydrous Na_2SO_4, evaporated and the residue distilled. It had b.p. 112°/22 mm. Yield 124 g. (89%). The product was stable for considerable periods of time if kept in a sealed vessel.

Reaction of the ester hydrochlorides with benziminoethyl ether

The ester hydrochloride mixture from 5 g. of protein was dissolved in 3 ml. of water, and the solution adjusted to pH 3–4 by addition of a drop or two of conc. NH_4OH. A solution of benziminoethyl ether (10 g.) in ether (30 ml.) was then added and the mixture thoroughly shaken for 16–24 hr. Griffin and Tatlock's 'Microid' flask shaker was found to be very suitable for this purpose. The shaking was generally carried out in 250 ml. conical flasks. On a larger scale it was preferable to divide the ester hydrochloride mixture into several portions, each not greater than about 10 g. The shaker held four flasks at a time. At the end of the shaking sufficient water was added to dissolve the NH_4Cl which had crystallized out, the upper layer was separated and washed several times with fresh portions of water. It was often necessary to filter the reaction mixture after addition of water to remove amorphous material which prevented a clean separation of the layers. The ethereal layer was then dried over anhydrous Na_2SO_4, the ether evaporated and the residue distilled, first at about 20 mm. to remove excess of benziminoethyl ether and finally at 0·01 mm., the fraction of b.p. 90–135°/0·01 mm. being collected. This is termed the oxazoline fraction. Periodate analysis showed that it contained about 80% of the threonine originally present in the protein hydrolysate before esterification.

Hydrolysis of the oxazoline fraction: isolation of a mixture of benzoylserine and benzoylthreonine

The oxazoline fraction was refluxed for 30 min. with 8 parts (w/v) of aqueous-ethanolic N-NaOH, prepared by mixing equal parts of 2N-NaOH and ethanol. The solution was then cooled, diluted with about half its volume of water and the ethanol removed under reduced pressure. The solution was acidified to pH 1 with 5N-HCl and allowed to stand 4 hr. at room temperature. A pale yellow oil separated. The solution was then extracted six times with about an equal volume each time of ethyl acetate, and the extracts rejected. To the aqueous solution, 5N-NaOH was added until alkaline to phenolphthalein. The solution was then made weakly acid, evaporated to a small bulk and acidified (blue to congo paper) with conc. HCl. The benzoyl derivatives were obtained from the solution by six extractions with ethyl acetate. The organic extracts were combined and evaporated to dryness under reduced pressure. The yield of mixed benzoyl derivatives was about 0·35 g. from 5 g. of protein, a yield of approximately 35% (assuming a threonine content of 4% and a serine content of 6%). On a larger scale the yield was about 30%; for example, 11·84 g. of mixed benzoyl derivatives were obtained from 200 g. of casein.

Isolation of pure L-*threonine and* DL-*serine*

(*a*) *Using a starch column*

About 300 mg. of the mixture of benzoyl derivatives were refluxed 4 hr. with 3N-HBr (2 ml.) and evaporated to dryness under reduced pressure. The residue was redissolved in water (20 ml.), extracted with ether to remove benzoic acid and the aqueous solution evaporated again to dryness. The residue was dissolved in 95% ethanol (2 ml.) and a slight excess of conc. NH_4OH added to the hot solution. After standing overnight the amino-acid was collected and washed with ethanol. The yield from 5 g. of protein was about 80 mg. It was recrystallized from a minimum of aqueous ethanol to remove traces of NH_4Br.

Synge (1944) used unfractionated potato starch in his experiments with *n*-butanol as solvent. In the experiments about to be described phenol saturated with water was used as the solvent. It was found necessary to remove small particles from the starch by sedimentation in water until the granules, when viewed under the microscope, appeared to be of approximately equal size. This represented only about 20% of the original amount of starch. The dried product was then washed several times by stirring with aqueous phenol and separating the liquid by centrifugation. It was then allowed to soak in aqueous phenol for several days. The starch was finally made into a chromatogram 25 cm. long and 1·1 cm. in diameter. The experiment was carried out in a room with the temperature controlled to about 70° F. The column was washed by allowing aqueous phenol to percolate through it for 4 days.

About 50 mg. of the serine-threonine mixture, dissolved in aqueous phenol, were added to the column, and when the solution had run through the column was developed

with aqueous phenol. The progress of the separation was followed by ninhydrin tests on a few drops of effluent. Threonine appeared in the effluent after 2–5 days and came through in a relatively small amount of solvent (12 ml.; flow rate of column about 1 ml./hr.). When the ninhydrin reaction became blank or very weak the receiver was changed and washing continued to recover the serine. The amino-acid was isolated from the phenol solution by adding it to an equal bulk of water and extracting the phenol twice with several volumes of ether. The aqueous solution was then evaporated to dryness and the residue crystallized from 80% ethanol. It was found that 100 mg. of amino-acid mixture could be used on a column 25 × 1·8 cm. The yield of L-threonine from 5 g. of protein was 40–45 mg., representing about 20% of the amount present in the hydrolysate. Samples of amino-acid isolated in this way were found to be 98–100% pure threonine by periodate analysis. No other amino-acid could be detected by paper chromatography. DL-Serine isolated from the column was also pure.

(b) Using countercurrent distribution

Most of the benzoylserine was first removed from the mixture of benzoyl derivatives by dissolving 4 g. in ethanol (10 ml.) and dry ether (30 ml.), and adding freshly distilled β-phenylethylamine (2·37 ml.). After standing at 0° for 2 days the crystalline *DL-benzoylserine β-phenylethylamine salt* (2·27 g.) was collected. The filtrate from the salt was evaporated to dryness under reduced pressure, the residue was dissolved in a small quantity of water and the solution acidified to congo red. The solution was extracted six times with ethyl acetate and the combined extracts evaporated to dryness under reduced pressure to recover the crude benzoylthreonine.

Isolation of DL-*benzoylserine.* The β-phenylethylamine salt was recrystallized several times from ethanol and then had m.p. 151–152°. (Found: N, 8·7. $C_{18}H_{22}O_4N_2$ requires N, 8·5%.) It gave no depression in melting point on admixture with a synthetic sample of the salt. DL-Benzoylserine was obtained in good yield from the salt by dissolving it in hot water and acidifying the solution (congo red paper). The acid crystallized on cooling and had m.p. 166–167°. (Found: N, 6·5. $C_{10}H_{11}O_4N$ requires N, 6·7%.) A 5% solution in ethanol was without effect on polarized light. About 7 g. of the β-phenylethylamine salt were obtained from 200 g. of casein.

Isolation of L-*threonine*

Method (i). *Ether/water system.* About 500 mg. of the crude benzoylthreonine were submitted to a 'diagonal stage' countercurrent distribution in twelve separating funnels using 50 ml. of ether and 7·5 ml. of water in each equilibration ($K_a = 0{\cdot}218$, $K_b = 0{\cdot}100$). The pooled ether layers were then evaporated to dryness and the residue washed with light petroleum (b.p. 60–80°). The solid was recrystallized several times from a small quantity of hot water to give 150 mg. of pure L-benzoylthreonine, m.p. 143–144°, $[\alpha]_D^{22°} + 26{\cdot}8°$ in water (*c*., 0·7). (Found: N, 6·2. Calc. for $C_{11}H_{13}O_4N$: N, 6·3%.) A synthetic specimen of L-benzoylthreonine, prepared by the method of West & Carter (1937), also had the same melting point and no depression in melting point was produced on mixing the two samples. West & Carter gave $[\alpha]_D + 25{\cdot}1$ for their product, whereas the synthetic sample prepared in this laboratory had $[\alpha]_D^{21°} + 27{\cdot}4°$, $+ 26{\cdot}9°$ in water (*c*, 0·7, 0·8). An additional amount of benzoylserine was isolated from the pooled aqueous layers as the β-phenylethylamine salt. Hydrolysis of the benzoylthreonine yielded pure L-threonine (found: threonine 100% by periodate analysis), which crystallized from 80% ethanol in hexagonal plates, characteristic of the pure amino-acid (see McCoy *et al.* 1935; Woolley & Peterson, 1937).

Method (ii). *Ethyl acetate/buffer system.* Phosphate-citrate buffer (2 M) was prepared by adding solid Na_2HPO_4 to 2 M-citric acid until pH 3·6 (glass electrode) was reached. The mixture was then diluted with sufficient water to make the solution 2 M with respect to phosphate plus citrate. About 4 g. of crude benzoylthreonine were submitted to a 10-funnel 'diagonal stage' countercurrent distribution between ethyl acetate and the buffer, using 200 ml. of the organic solvent and 164 ml. of the buffer for each equilibration ($K_a = 1{\cdot}39$, $K_b = 0{\cdot}48$). Except for the contents of the first funnel, in which most of the coloured impurities and some benzoic acid were found, the ethyl acetate layers were pooled, evaporated and the residue submitted to a second distribution as before. Citric acid was removed from the final product by a 4-funnel process using 200 ml. of ethyl acetate and 50 ml. of water for each equilibration. The purified benzoylthreonine was crystallized once from ethyl acetate giving a product m.p. 137–141°, which was hydrolysed with HBr to give L-threonine. The yield from 200 g. of casein was about 1·3 g. (16·5% of the amount present in the casein). The amino-acid was recrystallized once from aqueous ethanol to give about 0·9 g. of amino-acid, $[\alpha]_D^{20°} = -27{\cdot}8°$ in water (*c*, 5·0), purity by periodate analysis 96·3%. Another experiment, in which the benzoylthreonine was crystallized once from ethyl acetate before the second ethyl acetate-buffer distribution, gave a sample of amino-acid with $[\alpha]_D^{19°} - 28{\cdot}2°$ in water (*c*, 2·0), purity by periodate analysis 98·3%. West & Carter (1937) gave $[\alpha]_D^{26°} - 28{\cdot}3°$ for their synthetic sample; McCoy *et al.* (1935) found that the rotations of various isolated specimens varied from $-27{\cdot}5$ to $-28{\cdot}2°$.

Isolation of L-*serine from silk sericin by an alternative method*

A hydrolysate of silk sericin prepared from 50 g. of raw silk was esterified with *iso*propanol and the oxazoline fraction obtained in the usual way. The yield was 11·8 g. The main bulk (10·8 g.) of this fraction was shaken 3 hr. with 110 ml. of an acetate buffer prepared by adding solid sodium acetate to 20% acetic acid until pH 4 was reached (glass electrode). The oil which remained was extracted into ether and the extract thoroughly washed with water and $NaHCO_3$ solution. After evaporation of the ether the oil was refluxed 4 hr. with 5 N-H_2SO_4 (50 ml.); the solution was then extracted with ether to remove benzoic acid, diluted several times and the sulphate ions removed with $Ba(OH)_2$ in the usual way. Experiments on a small scale had revealed that the hydroxy-amino-acid N content of the solution as found by periodate NH_3 estimations was only 8% lower than the total amino N determined by nitrous acid. The filtrate and washings from the $BaSO_4$ were combined, evaporated to 60 ml. and *p*-hydroxyazobenzene-*p'*-sulphonic acid (4 g.) added to the hot solution. On cooling the serine salt separated in the form of orange plates. It was recrystallized from hot water giving 3·2 g. of pure salt. On decomposition of the salt by the method of Stein *et al.* (1942) 0·7 g. of pure L-serine was obtained. This crystallized in heavy hexagonal prisms from aqueous ethanol. It had $[\alpha]_D^{20°} - 7{\cdot}15°$ in water (*c*, 3·0), and was found to be 100% pure by periodate analysis.

SUMMARY

1. Reaction of an esterified protein hydrolysate with benziminoethyl ether gave a mixture of products containing the oxazolines derived from serine and threonine, and substituted imino ethers derived from amino-acid esters lacking the hydroxyl group in the β-position.

2. Complete separation of the oxazolines from the substituted imino ethers was not attempted because of the close proximity of the various boiling points. Alkaline hydrolysis of the oxazoline fraction, followed by a series of pH changes on the resulting solution gave a mixture of DL-benzoylserine and L-benzoylthreonine free from other amino-acid derivatives. The chemical and stereochemical changes involved are discussed.

3. Serine and threonine were separated from one another on a small scale by partition chromatography on a starch column and on a larger scale, as their benzoyl derivatives, by countercurrent distribution.

4. The yield of L-threonine obtained on a small scale was about 20% of the amount originally present in the protein.

5. L-Serine was isolated from silk sericin by an alternative method which was found not to be of general application.

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The Distribution of Ascorbic Acid in Developing Salmon (*Salmo salar* L.)

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Although attention has been focused upon physical and chemical changes that take place in the yolk and growing tissues of the developing salmon (and trout) egg, there are practically no references to vitamin content of the egg during these changes. The present investigation was limited to assays of ascorbic acid in two early embryonic stages, and one later, larval, stage of development in the salmon, and to its histological distribution. No estimations were made of the amounts of dehydroascorbic acid that might be present.

METHOD

The eggs were fertilized on 1 December 1947, and kept in running water at the following temperatures: 1–15 December 1948, 9–11°, average 9·9°. This covers the period for sample 1 (9 December) and sample 2 (15 December). 1 December–9 March 1948, 7–11°, average 9·0°. This covers the period from fertilization to the date of sample 3.

Fifty eggs or larvae were taken for each sample. The method followed was that worked out by Mapson (1943). Formaldehyde was used, as first recommended by Lugg (1942), to differentiate ascorbic acid from other substances

which might interfere with subsequent titration by 2:6-dichlorophenolindophenol. Mapson (1943) studied the effects of pH, concentration of formaldehyde and time, in order to distinguish titration values for sulphur-containing substances, ascorbic acid and certain non-specific substances (reductones) which occur in dehydrated food. The full method was applied to the present estimations, although it is not usual to find reductones in fresh tissue.

It is difficult to estimate ascorbic acid in different parts of the fertilized egg because of the semi-liquid nature of the yolk. It can be done, in a limited manner, if the yolk is first hardened by some fixative which also preserves the ascorbic acid. Consequently fifty eggs were taken, 19 days after fertilization, placed in a 4% (w/v) solution of HPO_3 in a closed vessel under N_2, and kept on ice for several hours. The blastoderm, together with the underlying periblast and a small amount of yolk, was then carefully removed from each egg by means of a razor-blade and treated as one sample. A second sample consisted of the rest of the yolk, yolk-sac and the protoplasmic vitellin membrane which lies between the outer membrane or chorion and the yolk. In order to handle these samples as quickly as possible they were not weighed.

For the histological demonstration of ascorbic acid in the stages examined, eggs and larvae were immersed in a 10% (w/v) solution of $AgNO_3$ in 10% (w/v) acetic acid in total darkness (Barnett & Bourne, 1942). The $AgNO_3$ solution penetrates the egg envelope slowly, and the yolk is so resistant that it has to be left in the solution for several hours. The tissue was subsequently fixed in $Na_2S_2O_3$ before bringing to the light and embedding in paraffin wax for section cutting.

RESULTS

With the two earlier samples the titrations showed no error due to the presence of sulphur compounds or reductones, since the values found by the method of Mapson (1943) were the same as those obtained by direct titration of a sample of the metaphosphoric acid extract with the indophenol reagent. The amount of ascorbic acid thus found was small. For the sample of 9 December it was 0·0106 mg./egg of average weight 153·3 mg., i.e. 140 i.u./100 g. For 15 December, it was 0·0103 mg./egg of average weight 157·4 mg., i.e. 131 i.u./100 g.

A different result, however, was obtained from a later sample taken on 9 March, when the larvae had been hatched 50 days and the yolk almost completely absorbed. After keeping the metaphosphoric acid extract in the dark at 20° for 1 hr. it was found that the titration value had fallen from 0·035 to 0·02 ml. of 2:6-dichlorophenolindophenol (strength 0·1 ml. equivalent to 0·04 mg. of ascorbic acid). According to Mapson (1943) such a fall is due to the presence of a substance similar to dihydroxymaleic acid. Further titrations at pH 0·6 gave the same result as that obtained in the blank. That is, no ascorbic acid was present, and the reading 0·02 ml. obtained in the preliminary stage was a measure of sulphur com-

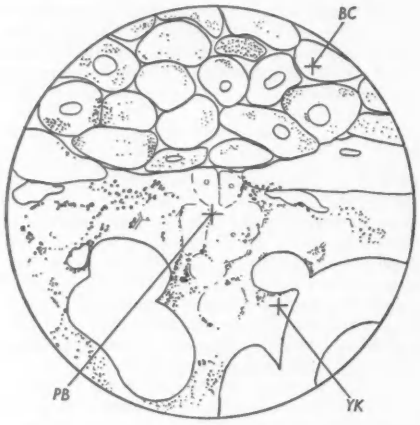

Fig. 1.

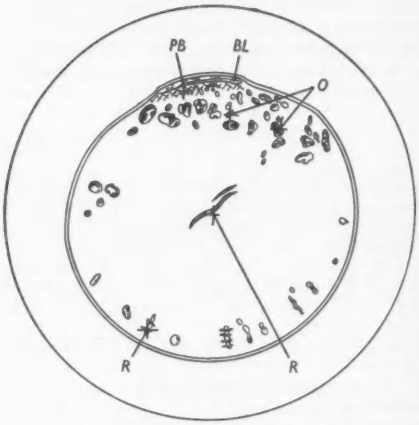

Fig. 2.

Fig. 1. Sagittal section through blastoderm cells, periblast and yolk of salmon egg 8 days after fertilization. Objective ⅓. Final magnification, ×288. The black dots and masses are presumed to indicate the distribution of ascorbic acid.

Fig. 2. Sagittal section of salmon egg 14 days after fertilization, showing position of blackened oil globules. Objective A.A. Final magnification, ×9·4. *BC*, blastoderm cell; *BL*, blastoderm; *O*, oil globules; *PB*, periblast; *R*, rifts in yolk; *YK*, yolk.

pounds which had reacted to formaldehyde at the particular hydrogen-ion concentration.

The method used, therefore, indicated that in the two earlier samples of fertilized salmon eggs ascorbic acid was the only substance present capable of reducing 2:6-dichlorophenolindophenol. In the advanced larvae of 9 March, however, reduction was brought about by material other than ascorbic acid.

The assumption that some ascorbic acid would be retained in the tissue when eggs were fixed in a 4% solution of metaphosphoric acid, before cutting away the blastoderm, was found to be correct. The metaphosphoric acid extract and the two samples were estimated separately with the following results:

Extract	0·41 mg. ascorbic acid from total wet weight of 7·432 g.
Blastoderm	0·123 mg. ascorbic acid.
Yolk and yolk sac	0·123 mg. ascorbic acid.

There was an equal residue of ascorbic acid in each sample but the yolk was the bulkier sample of the two. The results show that ascorbic acid was in the yolk and the yolk-sac as well as in the blastoderm and periblast, although the actual amounts are not indicated.

Examination of histological material showed that in both the early samples there had been reaction to the silver nitrate solution in the multicellular blastoderm. In prepared sections the majority of the cells contain blackened deposits of silver, either few in number or clustered together; they occur in the cytoplasm. It is not possible to connect them with yolk granules as appears to be the case in the chick (Barnett & Bourne, 1940–1); such granules are less obvious in blastoderm cells of the salmon. There was blackening, also, in the underlying protoplasmic layer of periblast. There were further deposits in the yolk immediate to the periblast where the texture is less homogeneous than in the central yolk (Fig. 1).

DISCUSSION

It is possible that substances in the tissue other than ascorbic acid reacted in a similar way to the silver nitrate solution. There is some agreement, however, between the general distribution of ascorbic acid in the egg as demonstrated by the titration method used, and the distribution of blackened granules of metallic silver in the prepared sections. It is assumed, therefore, that some of the granules, at least, result from ascorbic acid in the tissues.

Since there are no granular depositions in the yolk, other than those adjacent to the blastoderm, it may be assumed that the presence of the granules connected with activities of the blastoderm cells. This part of the yolk would be included in the estimation designated 'blastoderm'. Barnett & Bourne (1940–1) report a like deposition in the chick, and a similar laying down of material in yolk adjacent to actively dividing cells was found in the glycogen content of developing salmon (Daniel, 1946).

Although it has been demonstrated, by titration, that ascorbic acid is also present in the rest of the yolk-sac, it is difficult to form an opinion from histological preparations about its distribution. There is a general tendency for metallic silver to be precipitated wherever there are rifts or interfaces in the yolk, even in tissue that has been fixed for short periods (Fig. 2). Incidentally, there are similar deposits in sections that were passed through water and ethanol before being immersed in silver nitrate solution. They occur also in the late larval stage of 9 March, although quantitative analysis indicated that no ascorbic acid was present.

It is possible that masses of blackened material which lie in many oil vacuoles of the yolk may be connected with the presence of ascorbic acid, since the latter is not manifest in the surrounding yolk itself. Conditions under which a water-soluble vitamin, such as ascorbic acid, could be allied with oil globules call for further examination. In the meantime, two possibilities are presented:

(1) *Attraction of ascorbic acid to the oil globules.* This suggestion is prompted by an experiment of Barnett & Fisher (1944) with 5% gelatin, drops of olive oil and ascorbic acid to give a concentration of 200 mg./100 ml. The mixture was emulsified and pieces left in acidified silver nitrate for 10 min. Examination under the microscope showed that some of the oil globules had a heavy precipitate of metallic silver attached to the surface. The investigators claimed that this localization is a physicochemical phenomenon occurring after the precipitation. Their figure, however, shows that the blackening is almost wholly confined to the surface of the oil globules, and is different in kind from that found under somewhat similar conditions in yolk oil globules (Fig. 2).

(2) *Affinity between cholesterol and ascorbic acid.* Recent work on the adrenal gland has suggested an affinity between cholesterol and ascorbic acid (Long, 1947). It has been put forward by Zwemer, Lowenstein & Pines (1940) that the adrenal gland contains water-soluble steroids which are associated with ascorbic acid. Long states that approximately 90% of adrenal cholesterol is present in ester form. According to M. Scott (unpublished), only free cholesterol is present in oil obtained from yolk of developing salmon larvae. Conditions therefore would appear to be even more favourable for a linkage with ascorbic acid.

Finally, there may have been reaction between the silver nitrate solution and the original oil content of the vacuoles; in fact the amount of material involved suggests this to be the case. Smears of highly

unsaturated cod-liver oil, which is free from ascorbic acid, shows an intense black deposit after immersion in acidified silver nitrate in the dark (although not *in situ* in the liver), and to a limited extent this is also true of oil removed from salmon yolk.

In the late larval stage of 9 March, the various organs are almost completely differentiated. Although no ascorbic acid was found by estimation at this stage, there appears to be some deposition of metallic salts in the tissue. In section the issue is confused by the presence of black pigment, but there are certain discrete deposits near the vertebral column and in cells of the yolk-sac.

SUMMARY

1. It was found by the titrimetric method of Mapson (1943) that ascorbic acid was present in early stages of developing salmon (8 and 14 days after fertilization), but absent in a late larval stage in which most of the yolk had been absorbed. It was contained both in the blastoderm region and in the yolk and yolk-sac.

2. Sections obtained from material fixed in acidified silver nitrate show that ascorbic acid may be present in blastoderm cells, in the underlying periblast and also in adjacent yolk. In this respect the distribution is much the same as in early stages of chick development as recorded by Barnett & Bourne (1940–1).

3. There are intense deposits of metallic silver where oil globules have been situated in the main part of the yolk. These may be due, in part, to ascorbic acid present in the yolk, although there is also evidence in favour of a reaction between the silver nitrate solution and the oil globules.

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Studies in Biochemical Adaptation. Effect of Diet on the Intestinal Phosphatase of the Rat

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Verzár & McDougall (1936) suggested that the absorption of glucose and fructose is accompanied by phosphorylation by the intestinal mucosa. Part of the evidence upon which this theory was based was that iodoacetate decreases the rate of absorption of glucose, and this evidence has been criticized by several later writers (e.g. Klinghoffer, 1938). Again, Verzár supposed that an adrenal cortical hormone was responsible for the phosphorylation, and this has been criticized (Cori & Cori, 1941). Nevertheless, most workers accept the view that phosphorylation of glucose and fructose is an important stage in their absorption.

It has also been suggested that fats are phosphorylated before absorption (e.g. Sinclair, 1929). Verzár & McDougall (1936) claimed that this was controlled by the adrenal cortex, but, as with their theory of carbohydrate absorption, this view has since been severely criticized. Frazer (1948; see also Frazer, 1946) has suggested that fat may be absorbed as the unchanged triglyceride, although this is not universally accepted (Lundbaek & Maaløe, 1947). Most workers are agreed, however, that at least some phosphorylation of fat occurs during absorption (Schmidt-Nielsen, 1946).

It might be considered that diets with varying proportions of carbohydrate and fat might possibly stimulate the small intestine to produce varying amounts of phosphatase, since it is known that the amounts of some digestive enzymes are affected by variations in the degree to which they are called upon to carry out their work. A study was therefore

made of the amount of intestinal phosphatase in animals fed on diets containing different amounts of carbohydrate and of fat.

EXPERIMENTAL

Piebald rats of the Hartwell strain were placed on one of four diets for 6–7 months from weaning. The diets were constructed so as to contain varying proportions of protein, carbohydrate and fat (Table 1). Each diet was fed to ten rats, five male and five female, each rat being housed in a separate cage.

Table 1. *Composition of diets*

Diet	Casein ('Light white' Glaxo) (%)	Sucrose (%)	Lard (%)
Basal	15	55	15
Fat-free	15	70	0
High fat	15	0	70
High protein	70	15	0

In addition, each diet contained dried yeast (10%) and salt mixture (5%). Each animal was given cod-liver oil (5 drops twice weekly).

Preparation of intestinal extract. Animals were killed by exsanguination. The small intestine was dissected out, as free as possible from adherent tissue, from pylorus to caecum. The contents were removed by blowing a gentle stream of saline through the lumen. Since the activity of phosphatase decreases progressively from duodenum to caecum (Kay, 1932; Folley & Kay, 1936), it was thought desirable to assay the enzyme separately in the proximal and distal portions of the intestine. The intestine was therefore divided into as nearly as possible equal proximal and distal lengths. Each length was slit open, dried gently between filter paper and weighed. It was then ground with about twice its weight of washed silver sand. Water saturated with $CHCl_3$ was added to a volume exactly ten times the original wet weight of the intestine and the suspension kept for 24 hr. at laboratory temperature with occasional shaking.

Estimation of phosphatase activity. The alkaline phosphatase was assayed separately in the extracts from the proximal and distal parts of each intestine. The extracts, after standing for 24 hr., were further diluted with $CHCl_3$ water so that they contained 1 g. of wet tissue in 1000 ml. for the proximal part of the intestine and 1 g. in 250 ml. for the distal part. These dilutions were found to give enzymic activities such that conveniently measurable amounts of phosphate ions were liberated in the period of incubation with the substrate.

The phosphatase digest was set up as follows: 1 ml. of the diluted extract was added to 10 ml. of buffered sodium β-glycerophosphate (0·5 w/v), and the mixture incubated at 37° for 1 hr. Of this mixture, 1 ml. was added to 4 ml. of water; to this was added 5 ml. of trichloroacetic acid (12% w/v) and the whole well mixed. It was not necessary to centrifuge or filter at this stage since, owing to the high dilution of the enzymic preparation, the amount of protein precipitated was so little that no significant turbidity occurred.

Inorganic phosphate was estimated in the preparation by placing 5 ml. in a colorimeter tube, adding 4 ml. of acid molybdate reagent and 1 ml. of freshly prepared dilute $SnCl_2$ solution, the colour being measured in the Evelyn photoelectric colorimeter after 10 min. at laboratory temperature. An exactly similar analysis, carried out upon a 1 ml. sample of the digest taken immediately after adding the enzyme preparation, gave the initial inorganic phosphate content of the digest. The solutions of sodium β-glycerophosphate, molybdate and $SnCl_2$ were prepared as described by Bodansky (1932–3).

The activity of the enzyme is expressed in 'units' (mg. P liberated/g. tissue/hr. at pH 9·2 and 37°). No attempt was made to obtain possible activation of the enzyme by, for example, the addition of salts of Mg or Zn.

RESULTS

The mean weights of the animals and of the whole of their small intestines are given in Table 2. The differences between the different groups of animals

Table 2. *Effect of diet on weight of rat and of small intestine*

Diet	Average wt. of rats (g.)	Average wet wt. of small intestine (g.)
Basal	187	6·45
Fat-free	186	6·74
High fat	168	6·45
High protein	193	6·76
Average, all groups	184	6·62

are statistically not significant. As was to be expected, the average weights of the female animals and of their small intestines were lower than those of the males. Also, the average weights of the proximal portions of the intestines were higher than those of the distal portions. Table 3 shows these values for all groups combined.

Table 3. *Weight of rats and of small intestine. Comparison of male and female rats, and of proximal and distal portions of intestine*

	Average wt. of rats (g.)	Average wet wt. of small intestine (g.)		
		Whole	Proximal portion	Distal portion
Male	202	7·40	4·03	3·37
Female	166	5·83	3·17	2·66
Average, all rats	184	6·62	3·60	3·02

The values for phosphatase activities are given in Table 4. It seems reasonable to conclude that there is the same amount of intestinal phosphatase in the rats on the basal and fat-free diets and also the same amount in the rats on the high fat and high protein diets. On the other hand, there is more intestinal phosphatase in rats on either of the latter two diets than in those on either of the former two diets. For example, rats on the basal and fat-free diets had intestinal phosphatase values of 52 and 58 units/g.; rats on the high fat and high protein diets had values of 77 and 78 units/g.

Table 4. *Phosphatase activity of small intestine of rats on different diets*

(Ten rats on each diet. Results (mean ± S.E.) expressed as 'units', i.e. mg. P liberated in 1 hr. at pH 9·2 and 37°.)

Tissue		Diet: Basal	Fat-free	High fat	High protein
Whole intestine	Total units	(1) 330±46·5	(2) 391±41·7	(3) 489±39·3	(4) 538±42·6
	Units/g.	(5) 52±7·1	(6) 58±4·8	(7) 77±6·3	(8) 78±6·4
Proximal portion	Total units	(9) 271±40·7	(10) 328±31·3	(11) 375±33·0	(12) 448±37·2
	Units/g.	(13) 77±10·6	(14) 87±6·0	(15) 113±8·3	(16) 119±10·9
Distal portion	Total units	(17) 58±7·8	(18) 62±13·3	(19) 114±10·8	(20) 90±16·1
	Units/g.	(21) 20·3±3·4	(22) 20·3±3·9	(23) 34·5±3·5	(24) 29·0±3·7

Significant differences ($P < 0·05$) exist between the following groups: (1) and (3), (1) and (4), (2) and (4), (5) and (7), (5) and (8), (6) and (7), (6) and (8), (9) and (11), (9) and (12), (10) and (12), (13) and (15), (13) and (16), (14) and (15), (14) and (16), (17) and (19), (17) and (20), (18) and (19), (21) and (23), (22) and (23).

DISCUSSION

The chief difference between the basal and fat-free diets on the one hand and the high fat and the high protein diets on the other is the presence of much carbohydrate (55 or 70 % sucrose) in the former and its absence in the latter. It would seem therefore that the presence of sucrose leads to a reduction in the amount of phosphatase in the intestinal tissue. References to similar work are very meagre. Bellini & Cera (1940) found an increase in the intestinal phosphatase of the rat during fat absorption. Weil & Russell (1940) found a decrease in plasma phosphatase in the rat during fasting, and an increase during the feeding of lard and of cephalin but not of lecithin. Lundbaek & Goranson (1948) showed that the phosphorylase of rat muscle is increased during starvation.

We can at present suggest no reason why the presence of sucrose in the diet should lead to a decrease in intestinal phosphatase, and until more data are available it is profitless to speculate.

SUMMARY

The effect was studied of varying the proportions of dietary protein, fat and carbohydrate on the alkaline phosphatase of the small intestine of the rat. Diets containing 55 or 70 % of sucrose produced a significantly lower amount of the enzyme than diets in which the sucrose was replaced by fat or protein.

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The Reactions of Haems with Cyanides and *iso*Cyanides

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By determining the minimum amount of pyridine required to give a haemochromogen with a very strong solution of haem (ferrous protoporphyrin), Hill (1926) demonstrated conclusively that pyridine haemochromogen contains 2 molecules of organic base per molecule of haem or per atom of iron. This result was in agreement with that of von Zeynek (1910) who found 2·2 mol. of pyridine per mol. of haem in a solid pyridine haemochromogen.

Potassium cyanide, however, unlike other nitrogenous substances, was found by Anson & Mirsky (1928) to give two distinct compounds with haem. The first, containing 1 mol. of cyanide per molecule of haem, was named by them 'cyan-haemochromogen'

on account of the resemblance of its absorption spectrum to that of pyridine haemochromogen. The second compound, containing more cyanide per molecule of haem, was merely named the 'second cyanide compound'. The latter, which can only be obtained in presence of an excess of cyanide, differs from the first in the general pattern of its absorption spectrum. Unlike a typical haemochromogen its β-band is much stronger than its α-band. (Following general usage the letters α, β and γ denote the individual bands of the absorption spectrum of a compound, proceeding in that order from the red towards the ultraviolet regions of the spectrum. In haematin compounds the α- and β-bands are in the visible region; while the γ-band, also known as the Soret band, lies in the violet part of the spectrum.) These facts led Anson & Mirsky (1928) to consider all typical haemochromogens as compounds of haem with 1 mol. of a nitrogenous base, a conclusion which was not corroborated by later work. The nature of the compounds of cyanide with haem was reinvestigated by Hill (1929), who confirmed Anson & Mirsky's finding that haem and cyanide may combine in equimolecular proportions to give a compound with a haemochromogen-like spectrum, especially if the solution has a relatively low concentration of salts which tend to precipitate the haem out of solution. He also showed that the second compound, which had previously been obtained in the presence of excess cyanide, contains only 2 mol. of cyanide per molecule of haem, and he was able to determine the dissociation constants of both compounds.

As it is the 'second cyanide compound' of Anson & Mirsky (containing 2 molecules of cyanide per molecule of haem) that is structurally analogous to pyridine haemochromogen, Hill described it under the name 'cyan-haemochromogen' whereas for the monocyanide compound he proposed the name 'cyan-reduced-haematin'. Since, as will be shown later, both these cyanide-haem compounds differ in several respects from a typical haemochromogen, the protohaem compounds containing 1 and 2 mol. of cyanide will be described as monocyan-haem [(CN^-)-haem] and dicyan-haem [$(CN^-)_2$-haem] respectively. Suggested structures for these two compounds are

$$K^+ [\text{Porphyrin } Fe^{II} (CN)]^-$$

and $$K_2^{++} [\text{Porphyrin } Fe^{II} (CN)_2]^{--}.$$

In this paper it is proposed (1) to give the absorption spectra in the visible and violet regions of monocyan- and dicyan-haem; (2) to answer the question raised by Hill (1926) as to the existence of a compound in which the haem iron is linked simultaneously to the cyanide radical and carbon monoxide; (3) to elucidate the mode of attachment of the cyanide ion to the haem iron, since it is theoretically possible for the haem iron to combine with either the nitrogen or the carbon atom of the cyanide radical. For this the reactions of non-dissociable cyanides and *iso*cyanides with haems, haematins and methaemoglobin were examined.

EXPERIMENTAL

Protohaemin. This was prepared by the method of Schalfejeff (1885) from ox or horse blood.

Urohaemin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Other reagents. KCN solutions in distilled water were standardized either by the method of Robbie & Leinfelder (1945) or with standard $AgNO_3$ in the usual manner. Methyl cyanide was purified by adapting the method of Toda (1926) for the purification of butyl cyanide. Methyl *iso*cyanide was prepared according to the method of Gautier as modified by Hartley (1928).

Spectroscopic observations. For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The detailed study of absorption spectra was carried out with a Beckman photoelectric spectrophotometer using 0·5 and 0·25 cm. cells. The absorption coefficient is defined as $\epsilon = d/cl$, where c = molarity of the haematin solution, l = optical depth and d (density) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light, respectively.

RESULTS

Monocyan-haem

A stock solution of protohaematin ($1{\cdot}8 \times 10^{-4}$ M) was prepared by dissolving 11·7 mg. crystalline protohaemin in 100 ml. 1% (w/v) (anhydrous) Na_2CO_3. Monocyan-haem [(CN^-)-haem] was obtained by mixing 1 ml. of the stock haematin solution with 0·5 ml. of $3{\cdot}6 \times 10^{-4}$ M aqueous solution of potassium cyanide, the mixture being made up to 6 ml. with water and reduced with a few mg. of solid sodium dithionite ($Na_2S_2O_4$). In this solution the haem and cyanide were present in a 1:1 molecular ratio, the concentration of each being $0{\cdot}3 \times 10^{-4}$ M. The absorption spectrum of the scarlet solution of this compound shows two distinct bands in the visible region: α, 552 and β, 522 mμ., the α-band being much stronger than the β-band. The γ-band is at 414 mμ. The degree of dissociation which occurred under the conditions of this experiment was calculated from the value of the dissociation constant $K = 1{\cdot}3 \times 10^{-5}$ at 16°, given by Hill (1929). It was found that only 52·4% of the total haem was present as (CN^-)-haem, 47·6% remaining as free haem. These figures are in agreement with Hill's dissociation curve of (CN^-)-haem where cyanide and haem are present in a 1:1 molecular ratio. The absorption curve of (CN^-)-haem, as measured under the above experimental conditions, and the theoretical

curve of the absorption spectrum for 100% (CN^-)-haem, which was calculated from the observed values, are shown in Figs. 1 and 2.

The extinction coefficients of the α-, β- and γ-bands, measured and theoretical, are summarized in Tables 1 and 2.

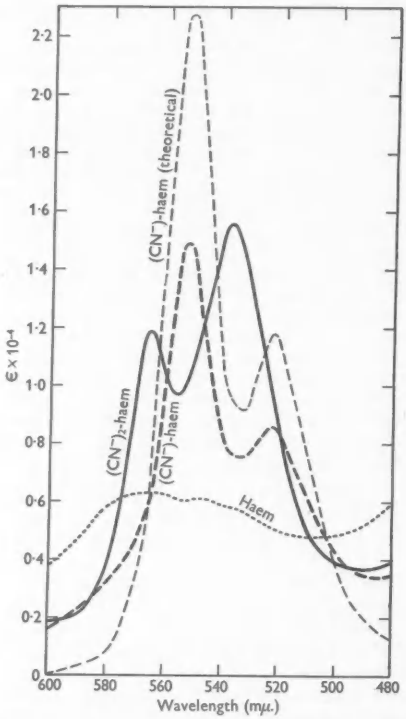


Fig. 1. Absorption bands in the visible region of protohaem, (CN^-)-protohaem and $(CN^-)_2$-protohaem. Haem $= 0{\cdot}3 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm., ϵ as defined in text. The theoretical absorption curve of (CN^-)-haem calculated for the undissociated compound is also given.

The absorption spectrum of this compound is of special interest in that although it is of a haemochromogen pattern, its α- and β-bands are considerably lower than those of typical haemochromogens obtained with denatured serum proteins, denatured globin, glycine or pyridine (Table 1). However, in the case of (CN^-)-urohaem (Fig. 5) it was found that the α-band was very nearly as high ($\epsilon \times 10^{-4} = 1{\cdot}99$) as that of a typical urohaemochromogen ($\epsilon \times 10^{-4} = 2{\cdot}23$ for glycine urohaemochromogen).

So far, the magnetic susceptibility of (CN^-)-haem has not been determined owing to technical difficulties. As has already been pointed out by Hill (1929), the strongest solution of (CN^-)-haem that can be obtained is 2×10^{-4} M. Even though the haem and cyanide are present in 1:1 ratio, a more concentrated solution would contain free haem in equilibrium with dicyan-haem. For the accurate determination of the magnetic susceptibility of a haematin derivative by the method of Gouy (1889) using a magnetic field of about 10,000 gauss, the concentration of the haem should be at least 10^{-2} M.

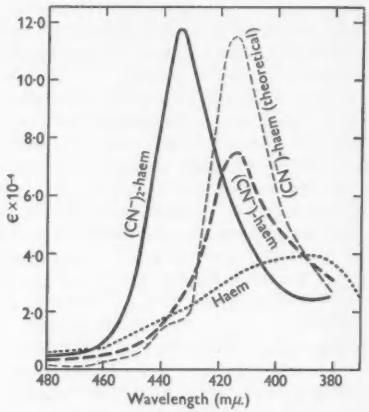


Fig. 2. Absorption spectra showing γ-bands of protohaem, (CN^-)-protohaem and $(CN^-)_2$-protohaem, and the theoretical absorption curve of (CN^-)-protohaem calculated for the undissociated compound. Haem $= 0{\cdot}3 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.

Dicyan-haem

Dicyan-haem, $[(CN^-)_2$-haem], was prepared by mixing 1 ml. of the stock haematin solution with 5 ml. of $3{\cdot}6 \times 10^{-3}$ M aqueous potassium cyanide solution reduced with the minimum amount of dry $Na_2S_2O_4$. The concentration of haem was $0{\cdot}3 \times 10^{-4}$ M and the haem:cyanide ratio was 1:100. The absorption spectrum of the solution of this compound, which is also scarlet, differs markedly from that of (CN^-)-haem (Figs. 1 and 2). The positions of its two absorption bands in the visible region of the spectrum are: α, 565 and β, 536 mμ., and the α-band is much weaker than the β-band, the values $\epsilon \times 10^{-4}$ being 1·185 and 1·56, respectively. The γ-band lies at 434 mμ. The extinction coefficients of the absorption bands are summarized in Table 2.

Reactions with carbon monoxide

Reactions of haemochromogens with carbon monoxide. It is well known that haem and haemochromogens combine reversibly with CO and that the CO-haem and CO-haemochromogens thus formed each contain only 1 mol. of CO/mol. of the compound

Table 1. *Positions and heights of the α- and β-bands and the trough between them for monocyan-protohaem and typical haemochromogen compounds*

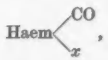
Compound	Band or trough (tr.)	Wavelength of maxima and minima of absorption (mμ.)	$\epsilon \times 10^{-4}$	$\frac{\epsilon_\alpha}{\epsilon_{tr.}}$	$\frac{\epsilon_\beta}{\epsilon_{tr.}}$
Monocyan-protohaem (theoretical value)	α	552	2·27		
	tr.	534	0·91	2·5	1·3
	β	522	1·18		
Denatured serum protein haemochromogen (Keilin, 1944)	α	557	2·96		
	tr.	540	0·79	3·76	1·64
	β	527	1·29		
Glycine haemochromogen (Keilin, unpublished)	α	556	3·02		
	tr.	539	0·83	3·65	1·71
	β	525	1·30		
Globin haemochromogen (Drabkin, 1942)	α	558	3·09		
	tr.	542	0·89	3·49	1·58
	β	528	1·40		
Pyridine haemochromogen (Drabkin, 1942)	α	558	3·09		
	tr.	540	0·91	3·41	1·79
	β	525	1·63		

Table 2. *Positions and extinction coefficients of the α-, β- and γ-bands of the compounds of protohaem and urohaem with* KCN, CH_3NC *and* CO

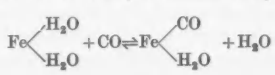
Compound	α-Band λ (mμ.)	α-Band $\epsilon \times 10^{-4}$	β-Band λ (mμ.)	β-Band $\epsilon \times 10^{-4}$	γ-Band λ (mμ.)	γ-Band $\epsilon \times 10^{-4}$	$\frac{\epsilon_\gamma}{\epsilon_\alpha}$
(CN^-)-protohaem (theoretical)	552	2·27	522	1·18	414	11·45	5
(CN^-)-protohaem (exp.)	552	1·49*	522	0·86*	414	7·5*	5
(CH_3NC)-protohaem	556	1·515*	528	1·13*	420	10·45*	7
$(CN^-)_2$-protohaem	565	1·185	536	1·56	434	11·75	10·0
$(CH_3NC)_2$-protohaem	562	1·245	532	1·415	432	14·48	11·6
CO-protohaem	562	1·46	530	1·195	406·5	14·7	9·6
(CN^-)-protohaem + CO	562	1·40	530	1·15	406·5	14·1	10·0
CO-cyan-protohaem	565	0·97	538	1·185	425	15·0	15·4
CO-protohaem (in ethanol)	562	1·185	533	0·92	410	15·6	13·2
CO-(CH_3NC)-protohaem	564	1·205	536	1·155	422·5	10·0	8·3
(CN^-)-urohaem	547	1·99*	518	1·17*	418	11·2*	5·6
(CH_3NC)-urohaem	547	1·655*	516	1·185*	410	17·5*	10·5
$(CN^-)_2$-urohaem	560	0·83	532	1·46	428	11·0	13·25
$(CH_3NC)_2$-urohaem	554	1·1	524	1·55	422	26·8	24·5

* These values apply to the compound under the experimental conditions described and are much lower than the theoretical value owing to dissociation.

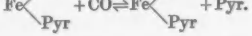
(Hill, 1926). According to Hill CO-haem can be represented as



'where x is either another molecule of the complex or a molecule of water'. (In the schematic representations of these compounds, the lines directed from the haem iron are not intended to indicate the nature of the valency bonds.) Using the formula for haem suggested by Davies (1940) the reaction may be expressed as follows (Fe = haem iron):



By passing CO through a solution of a haemochromogen, e.g. pyridine haemochromogen, the CO displaces 1 mol. of pyridine (Pyr) to form a CO-haemochromogen (Hill, 1926):



It is important to note that in this equilibrium reaction CO and pyridine compete for the haem iron.

Reaction of monocyan-haem with carbon monoxide. On passing CO through a solution of (CN^-)-haem, the latter acquires a characteristic bright cherry-red colour, and its absorption spectrum in both the visible and violet regions becomes almost

indistinguishable from that of ordinary CO-haem. To avoid loss of cyanide as CO is bubbled through the solution, the experiment was also carried out by adding an equivalent amount of cyanide to CO-haem previously prepared. However, the same result was obtained in both cases and the compound formed could not be distinguished from CO-haem. These results support the observation of Hill (1929) that when (CN^-)-haem is treated with CO the latter replaces the single cyanide ion giving CO-haem and not CO-(CN^-)-haem. The reaction may therefore be represented as follows:

$$Fe\begin{matrix}CN^- \\ H_2O\end{matrix} + CO \rightleftharpoons Fe\begin{matrix}CO \\ H_2O\end{matrix} + CN^-$$

Reaction of dicyan-haem with carbon monoxide. When CO was passed through a solution of $(CN^-)_2$-haem, the characteristic absorption bands of this compound in the visible region of the spectrum disappeared and were replaced by a diffuse and asymmetric band as seen in the microspectroscope. The spectrophotometric curve of this band (Fig. 3), however, showed it to consist of a two-banded spectrum with a very weak and narrow band at 565 mμ. and a strong band at 538 mμ. There was also a very marked change in the violet region of the spectrum; the γ-band of the compound became distinctly sharper and was shifted from 434 to 425 mμ., i.e. about 9 mμ. towards the short wave end of the spectrum (Fig. 4, Table 2). The absorption spectrum of this compound is thus very different from those of (CN^-)-haem, $(CN^-)_2$-haem, CO-haem and CO-haemochromogen. These results clearly demonstrate the existence of CO-cyan-haem, which may be considered as analogous to CO-haemochromogens:

$$Fe\begin{matrix}CN^- \\ CN^-\end{matrix} + CO \rightleftharpoons Fe\begin{matrix}CO \\ CN^-\end{matrix} + CN^-.$$

Carbylamine haems

Reactions of methyl iso*cyanide with haems.* Pure methyl *iso*cyanide (CH_3NC) was dissolved in distilled water to make a 0·15 M solution. A 10^{-3} M solution of protohaematin was prepared by dissolving 16·4 mg. of crystalline protohaemin in 25 ml. 0·1 N-NaOH. The *iso*cyanide solution (1 ml.) was added to 1 ml. haematin solution, and the mixture was diluted with methanol and distilled water so that the final methanol concentration was at least 50 %. When this solution, containing haem and *iso*cyanide in a 1:150 molecular ratio, was reduced with $Na_2S_2O_4$ the red solution showed a two-banded spectrum resembling that of $(CN^-)_2$-haem, the β-band being sharper than the α-band. The positions of the bands were: α, 562, β, 532 and γ, 432 mμ., the values of $\epsilon \times 10^{-4}$ being 1·25, 1·42 and 14·48, respectively (Table 2). A similar experiment using equimolecular (10^{-3} M) solutions of haematin and *iso*cyanide gave rise to a spectrum resembling that of (CN^-)-haem in

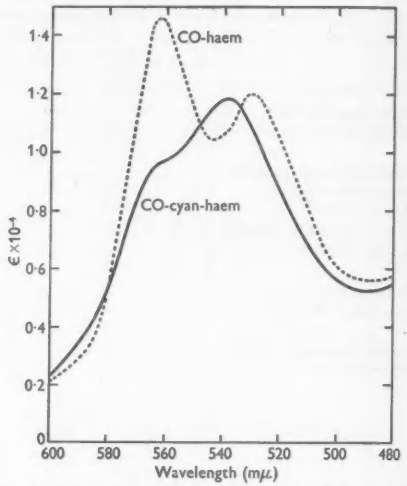


Fig. 3. Absorption bands in the visible region of the spectrum of CO-protohaem and CO-cyan-protohaem. Haem = $0·3 \times 10^{-4}$ M, $l = 0·5$ cm.

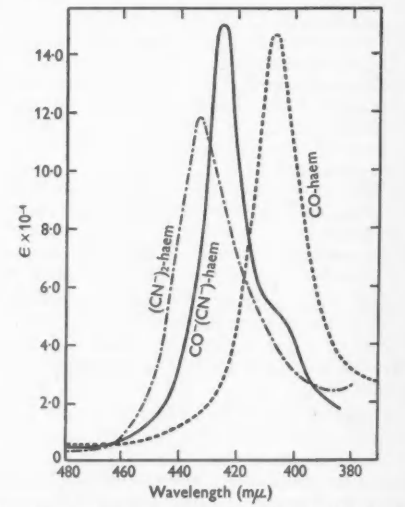


Fig. 4. Absorption spectra showing γ-bands of CO-protohaem, $(CN^-)_2$-protohaem and CO-cyan-protohaem. The shoulder at about 405 mμ. on the latter absorption band is probably due to a little CO-haem in the solution. Haem = $0·3 \times 10^{-4}$ M, $l = 0·5$ cm.

which the α-band was much sharper than the β-band. Owing to some dissociation under the conditions of the experiment the absorption spectrum is

probably that of a mixture of (CH_3NC)-haem with a little free haem and $(CH_3NC)_2$-haem, the latter giving rise to a low γ-band in addition to that of (CH_3NC)-haem. The positions and extinction coefficients of the absorption bands of this solution are summarized in Table 2.

With urohaem, CH_3NC reacts freely in slightly alkaline solution, presumably because urohaem is so much more soluble than protohaem. Two compounds are formed as with protohaem, and the spectra are

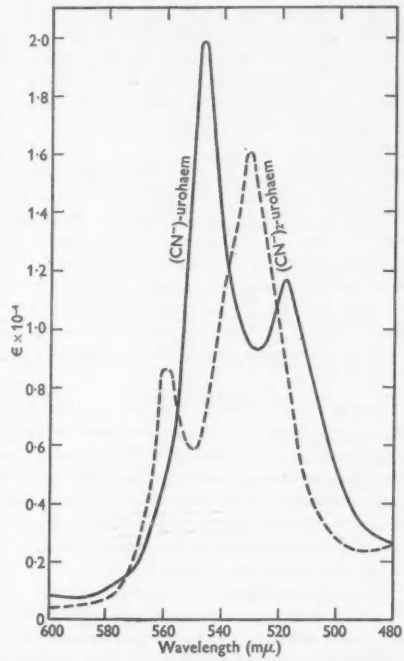


Fig. 5. Absorption bands in the visible region of the spectrum of (CN^-)-urohaem (Haem:KCN = 1:3·6) and $(CN^-)_2$-urohaem, containing excess KCN. Urohaem = $0{\cdot}55 \times 10^{-4}$ M for first compound and $0{\cdot}661 \times 10^{-4}$ M for second compound; $l = 0{\cdot}25$ cm. The γ-bands of these compounds are shown in Fig. 7.

very similar to those of mono- and di-cyan-urohaem. (CH_3NC)-urohaem has its α-, β- and γ-absorption bands at 547, 516 and 410 mμ., the values of $\epsilon \times 10^{-4}$ being 1·66, 1·19 and 17·5, respectively (Figs. 6 and 7).

$(CH_3NC)_2$-Urohaem has α- and β-bands at 554 and 524 mμ., the values of $\epsilon \times 10^{-4}$ being 1·1 and 1·55, respectively. The γ-band of this compound, which lies at 422 mμ., is of interest in that the value of $\epsilon \times 10^{-4}$ is about 26·8 (certainly above 25·6) (Figs. 6 and 7). The only other metalloporphyrin known which possesses such a high γ-band is turacin, a naturally occurring copper-uroporphyrin. In turacin the γ-band is at 399 mμ. and $\epsilon \times 10^{-4}$ is 26.

The reaction between protohaem and 10^{-2}M-CH_3NC in alcoholic solution with cysteine buffer as reducing agent was described by Warburg, Negelein & Christian (1929) in the course of their investigations on the compound formed between methyl *iso*cyanide and haemoglobin and on the effects of methyl *iso*cyanide on the photochemical dissociation of CO-haemoglobin. These authors give the absolute absorption spectrum of methyl carbylamine-haem of which only the γ-band resembles that of $(CH_3NC)_2$-urohaem as shown in Fig. 7. The mono-carbylamine-haem compound was not described by these authors.

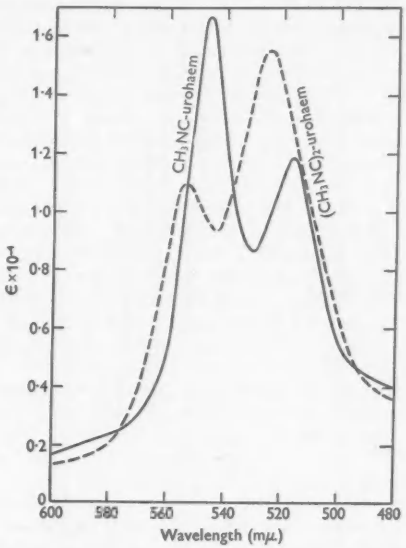


Fig. 6. Absorption bands in the visible region of the spectrum of CH_3NC-urohaem (urohaem:CH_3NC = 1:1) and $(CH_3NC)_2$-urohaem (urohaem:CH_3NC = 1:150). Urohaem = $0{\cdot}834 \times 10^{-4}$ M, $l = 0{\cdot}25$ cm.

Reactions of carbon monoxide with mono- and di-carbylamine haems. With (CH_3NC)-protohaem, as in the case of (CN^-)-protohaem, CO displaces the single molecule of CH_3NC from the haem Fe, and ordinary CO-haem is obtained. When CH_3NC was added to an ethanolic solution of CO-protohaem so that the molecular ratio of CH_3NC to haem was 3:1, the α- and β-bands became more diffuse and a γ-band appeared at 422·5 mμ. in addition to that of CO-haem (410 mμ.). This new band, which lay in a position intermediate between those of CO-haem and $(CH_3NC)_2$-haem, was most probably due to a CO-carbylamine-haem compound, analogous to CO-(CN^-)-haem. The same result is obtained if CO is

passed through a solution of $(CH_3NC)_2$-haem containing CH_3NC and haem in the same 3:1 ratio. If more CH_3NC is present, e.g. 15 mol./mol. of haem, the CO compound does not appear to be formed.

Reactions of methyl cyanide with haem. Whereas a commercial sample of methyl cyanide (CH_3CN) reacted with urohaem giving a 4-banded spectrum corresponding to a mixture of the mono- and di-cyan-haem types of spectra, a pure specimen (b.p. 81°) which had been treated with N-H_2SO_4 to hydrolyse any *iso*cyanide present, followed by N-NaOH to remove any free HCN, failed to react with urohaem in any proportions in both aqueous and ethanolic solutions. The importance of this failure to react with haem will be discussed later.

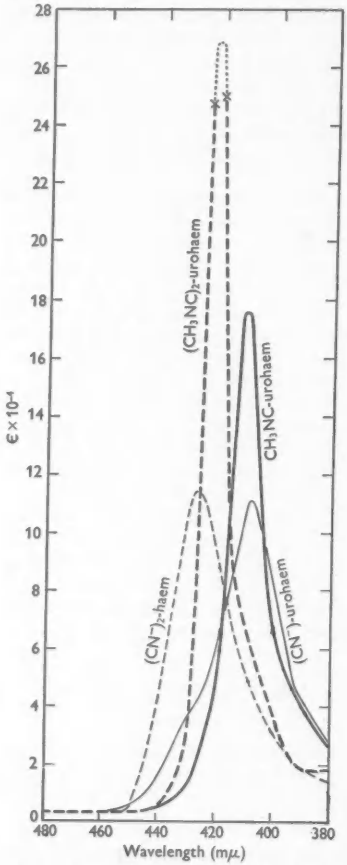


Fig. 7. Absorption spectra showing γ-bands of $(CN^-)_2$-urohaem, (CN^-)-urohaem (solutions as in Fig. 5), $(CH_3NC)_2$-urohaem and CH_3NC-urohaem (solutions as in Fig. 6). $l = 0{\cdot}25$ cm.

Reactions of methyl isocyanide and methyl cyanide with the trivalent iron compounds, haematin and methaemoglobin

Haematin. KCN reacts readily with haematins causing the colour to change from greenish brown to scarlet, and the narrow absorption band at about 615 mμ. of alkaline protohaematin to be replaced by a broad, somewhat diffuse band in the green region of the spectrum at 545 mμ. According to Hogness, Zscheile, Sidwell & Barron (1937) cyanhaematin contains 2 mol. of cyanide/mol. of haematin. If, however, CH_3NC or CH_3CN is added to protohaematin in ethanolic solution, there is no change in the haematin spectrum, an indication that neither of these compounds reacts with haematin. Similar negative results were obtained with urohaematin.

Methaemoglobin. KCN also combines with acid methaemoglobin, altering the colour of the solution from reddish brown to bright red and causing the absorption band at 630 mμ. to be replaced by a broad band the centre of which lies at about 540 mμ. Neither methyl *iso*cyanide nor methyl cyanide causes any change in the colour or spectrum of methaemoglobin, and it may therefore be assumed that these compounds do not combine with it.

DISCUSSION

Haemochromogens have hitherto been defined as compounds of haem with 2 mol. of a nitrogenous base. Potassium cyanide and methyl *iso*cyanide, however, differ from all other nitrogenous substances investigated so far, in that each can form with haem two distinct compounds containing 1 and 2 mol. of the substance per molecule of haem.

In his study of the two cyanide-haem compounds, Hill (1929) found that on the addition of CO to (CN^-)-haem, ordinary CO-haem was formed and not a CO-(CN^-)-haem compound, the single cyanide ion being replaced by CO. He stated, however, that 'the existence of a compound such as CO-cyan-haemochromogen is not excluded, but so far it has not been detected'. It is now shown that there is ample spectroscopic evidence for the existence of a definite CO-(CN^-)-haem. The conditions required for its formation show that in the presence of excess cyanide, i.e. when $(CN^-)_2$-haem is formed, one of the CN^- groups of this compound can be replaced by CO giving CO-(CN^-)-haem. Apparently in (CN^-)-haem, CO can replace only the (CN^-)-group and not the water co-ordinated with the iron atom, as shown in the scheme on p. 444.

The CO-(CN^-)-haem is of special interest since it shows that two important inhibitors of respiration and of reactions catalysed by certain haematin compounds may, under certain conditions, co-ordinate simultaneously with the same iron atom of haem.

A comparative study of the properties of the two cyanide-protohaem compounds and ordinary haemochromogens (e.g. globin or pyridine haemochromogen) shows that: (1) (CN^-)-haem has an absorption spectrum of the same pattern as that of a haemochromogen but, unlike the latter, it contains only 1 mol. of nitrogenous base/atom of Fe; its absorption bands both in the visible and violet regions of the spectrum are considerably lower than those of a haemochromogen, and it does not form a CO compound of the CO-haemochromogen type. Unfortunately, its magnetic susceptibility has not yet been determined, for it would be very interesting to know whether or not the compound is diamagnetic as suggested by its absorption spectrum. (2) $(CN^-)_2$-haem resembles a haemochromogen in its composition (2 mol. of nitrogenous substance per atom of Fe), in the zero magnetic moment of the Fe atom (Pauling & Coryell, 1936) and in forming a CO-(CN^-)-haem compound analogous to a CO-haemochromogen, but it differs from a haemochromogen in several ways. The patterns of the absorption spectra of $(CN^-)_2$-haem and its CO derivatives are entirely different from those of a haemochromogen and CO-haemochromogen respectively and, in addition, the densities of the absorption bands of $(CN^-)_2$-haem are only about half of those of a typical haemochromogen.

Methyl *iso*cyanide has now been shown to give two compounds with haem analogous to those given by KCN. Since methyl *iso*cyanide (CH_3NC) is only able to combine with the haem Fe by means of its terminal carbon atom, it may be assumed that in both mono- and di-cyan-haem the cyanide ion is also linked by its carbon atom. Additional evidence in favour of this view is provided by the inability of methyl cyanide (CH_3CN) to react with haem either in the manner of KCN or as a simple haemochromogen-forming base in spite of its terminal nitrogen atom. These results corroborate the views of Pauling & Coryell (1936) and Wyman (1948) that 'in the dicyanide compound it is probably the carbon atoms of the cyanide ions which are linked with the iron'. It is this mode of linkage which is probably responsible for the fundamental differences between the cyanide and carbylamine haem compounds on the one hand and the true haemochromogens on the other. In the latter type of compound the nitrogenous base is always linked to the haem Fe through the nitrogen atom.

It is of interest to note that methyl *iso*cyanide reacts with the bivalent iron of haem and haemoglobin but not with the tervalent iron of haematin and methaemoglobin. In this respect methyl *iso*cyanide resembles CO, with which it can compete for ferrous haem and haemoglobin.

The structure of dicyan-haem is of particular interest since the pattern of its absorption spectrum bears a strong resemblance to a certain haem derivative which was first described by Dhéré & Vegezzi (1916) and which will be discussed in the next paper.

SUMMARY

1. As was previously shown, potassium cyanide gives rise to two distinct compounds with haem: monocyan-haem (1 CN^-/Fe) and dicyan-haem (2 CN^-/Fe).

2. The absorption curves of these compounds have been determined in the visible as well as the violet regions of the spectrum and the marked differences in their pattern are discussed in relation to the spectra of other haematin derivatives.

3. Methyl *iso*cyanide (CH_3NC) combines with haems to give mono- and di-carbylamine-haem compounds having the same spectroscopic properties as mono- and di-cyan-haems, whereas methyl cyanide (CH_3CN) does not combine with haems. These results show that the cyanide ions in mono- and di-cyan-haems are attached to the haem iron through their carbon atoms. This mode of attachment accounts for the difference between the absorption spectra of $(CN^-)_2$-haem or $(CH_3NC)_2$-haem and those of ordinary haemochromogen compounds such as pyridine haemochromogen.

4. It is only when one cyanide ion or one molecule of methyl *iso*cyanide is attached to the haem iron through its carbon atom that the absorption spectrum of the resulting compound resembles that of an ordinary haemochromogen.

5. Methyl *iso*cyanide, like carbon monoxide, combines only with haem and haemoglobin and does not react with the ferric compounds haematin and methaemoglobin, both of which form characteristic compounds with cyanide ion.

6. Methyl cyanide was found not to react with any of the haematin compounds examined.

7. The existence of a CO-(CN^-)-haem compound in which iron is simultaneously co-ordinated with cyanide and carbon monoxide has been demonstrated, and an analogous CO-(CH_3NC)-haem compound is also described.

I wish to thank Dr R. Hill, F.R.S., and Dr E. F. Hartree for their valuable suggestions, Prof. C. H. Gray for putting me in contact with a case of congenital porphyria without whose ready co-operation much of this work could not have been carried out, and Mr C. L. Tsou for the preparation of a sample of methyl *iso*cyanide. Thanks are also due to the Medical Research Council for a personal grant.

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On the Properties and Nature of Dihydroxyl-haem

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Dhéré & Vegezzi (1916), while working on the reduction of haematin under different conditions, described a compound obtained by reducing an ethanolic solution of haematin in strong alkali with sodium dithionite ($Na_2S_2O_4$) in air. This compound, which they named 'alkaline haemochromogen', was red in colour and showed a characteristic absorption spectrum consisting of two bands, of which the β-band lying nearer the blue end of the spectrum was much stronger than the α-band. It was not until several years later, however, that the term 'haemochromogen' was defined by Anson & Mirsky (1925) as covering the compounds of reduced haematin or haem with different nitrogenous substances, and Hill (1926) showed conclusively that in a haemochromogen 2 mol. of an organic base are combined with the one iron atom of haem. Of all the nitrogenous compounds examined only potassium cyanide and methyl *iso*cyanide (methyl carbylamine) were found to form two distinct compounds with haem: monocyan-haem and dicyan-haem, which contain 1 and 2 mol. of cyanide per molecule of haem respectively, and mono- and di-carbylamine haem (see Keilin, 1949).

The object of this investigation is to elucidate the nature of the compound described by Dhéré & Vegezzi as 'alkaline haemochromogen' and to compare it with haem, monocyan-haem, dicyan-haem and haemochromogens. It is proposed to describe the 'alkaline haemochromogen' of Dhéré & Vegezzi under the name of dihydroxyl-haem, with a prefix to the haem denoting the type of porphyrin used; e.g. dihydroxyl-protohaem and dihydroxyl-urohaem.

MATERIAL AND METHODS

Protohaemin. This was prepared by the method of Schalfejeff (1885) from ox or horse blood.

Haematohaemin. Haematoporphyrin was prepared from protohaemin by Nencki's method (Nencki & Seiber, 1888) and the iron was introduced by treating the porphyrin with $FeSO_4$ in the presence of glacial acetic acid and sodium acetate.

Urohaemin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Spectroscopic observations. For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The recording of absorption spectra in the visible and violet regions was carried out with a Beckman photoelectric spectrophotometer. In all figures the molecular absorption coefficients (ϵ), as previously defined (Keilin, 1949), were plotted against wavelengths (in mμ.).

RESULTS

The reaction of protohaem with sodium hydroxide

A solution of protohaem, obtained by dissolving protohaemin in 0·1 N-NaOH or 1% (w/v) Na_2CO_3 (anhydrous) and reducing it with $Na_2S_2O_4$, shows two very diffuse absorption bands. The addition of ethanol to this solution affects this spectrum only in so far as it causes a very slight sharpening of the bands. If, however, the ethanolic solution of protohaematin contains a much higher concentration of NaOH, the solution becomes cherry coloured on reduction, and a characteristic two-banded spectrum is seen in which the β-band, with its centre at

558 mμ., is much stronger than the α-band which lies at 590 mμ. (Fig. 1). The γ-band of this compound

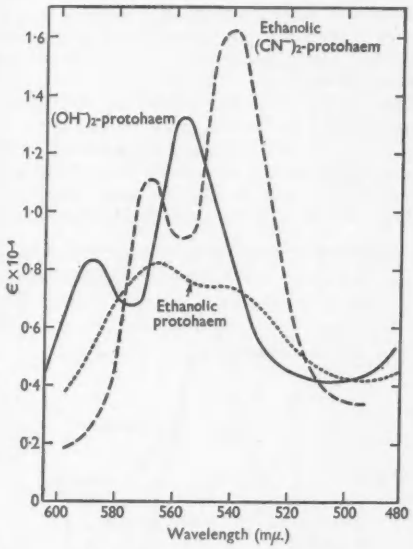


Fig. 1. Absorption bands in the visible region of the spectrum of $(OH^-)_2$-protohaem, $(CN^-)_2$-protohaem containing 50% v/v ethanol (protohaem $= 1{\cdot}85 \times 10^{-5}$ M) and ethanolic protohaem. (Protohaem $= 1{\cdot}65 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.)

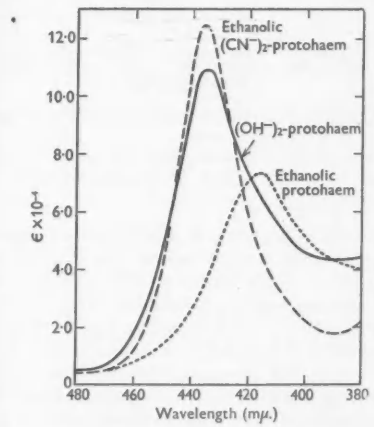


Fig. 2. Absorption spectra showing the γ-bands of $(OH^-)_2$-protohaem, ethanolic protohaem and $(CN^-)_2$-protohaem containing 50% v/v ethanol. (Protohaem $= 1{\cdot}85 \times 10^{-5}$ M, $l = 0{\cdot}5$ cm.)

($\lambda = 435$ mμ., $\epsilon \times 10^{-4} = 11$) is much stronger and lies nearer the red end of the spectrum than the corresponding band ($\lambda = 412$ mμ., $\epsilon \times 10^{-4} = 7{\cdot}2$) of ethanolic protohaem which, for the purposes of this paper, may be defined as a solution of haematin in a 50% (v/v) mixture of 0·1 N-NaOH and ethanol, reduced with $Na_2S_2O_4$ (Fig. 2). Dihydroxyl-protohaem can also be obtained if the ethanol is replaced by acetone or glycol monomethyl ether. The effect of ethanol is therefore not specific, and the formation of this compound requires the presence of almost any water-miscible solvent in addition to a high concentration of NaOH.

The optimum conditions for the formation of dihydroxyl-protohaem were determined by varying the concentration of (*a*) ethanol or (*b*) NaOH, the concentrations of the other reagents remaining constant in both cases.

(*a*) *Experiments with varying concentrations of ethanol.* For this purpose a series of tubes was set up each containing 1 ml. of a $4{\cdot}6 \times 10^{-4}$ M solution of protohaematin in 1% (w/v) Na_2CO_3 and 1 ml. of 10% (w/v) NaOH. To all except the first tube were added varying quantities of absolute ethanol. The solutions were made up to a constant volume of 4 ml. with distilled water, reduced with dry $Na_2S_2O_4$ and examined spectroscopically.

The solutions containing 28·5% (v/v) or less of ethanol were opalescent, of a greenish brown colour and showed the spectrum of ordinary protohaem. When the concentration of ethanol reached 34% (v/v) the absorption spectrum of dihydroxyl-protohaem was faintly seen, superimposed on that of haem. With increasing concentrations of ethanol, the absorption bands of dihydroxyl-protohaem gradually replaced those of protohaem. The bands reached maximal intensity and the solution became clear and of a brownish pink colour only when it contained about 45% (v/v) ethanol. As a slightly higher concentration of ethanol did not affect the absorption spectrum of the compound, it was decided to add ethanol to 50% of the total volume of the solution in all subsequent experiments with dihydroxyl-protohaem.

(*b*) *Experiments with varying concentrations of sodium hydroxide.* In an attempt to determine whether the formation of the dihydroxyl-protohaem was a function of the pH of the solution, Na_2CO_3 was used as the alkali, but even saturation of a solution of ethanolic protohaem with Na_2CO_3 failed to produce the characteristic spectrum. It was found that only strong alkali such as NaOH would give rise to this spectrum and the minimal concentration for its complete formation was determined spectrophotometrically as follows. A stock solution of protohaematin was prepared containing 12·35 mg. protohaemin in 25 ml. 1% (w/v) Na_2CO_3 and a series of 10 tubes was set up each containing 1 ml. of the stock haematin solution and 3 ml. absolute ethanol. To tubes 2–10 were added varying amounts of

NaOH ranging from 0·5 ml. N-NaOH to 2 ml. 12N-NaOH and the volumes of all solutions were adjusted to 6 ml. with distilled water. The solutions were reduced with solid $Na_2S_2O_4$ and allowed to stand for about 30 min. to allow full reduction to take place, especially in the more alkaline solutions. The maximum intensities of the absorption bands were measured for each solution, and it was found that a concentration of 1·33N-NaOH provided the optimum conditions for the formation of dihydroxyl-protohaem.

The choice of solvent used for the formation of dihydroxyl-protohaem. In the reaction described above ethanol was used as originally specified by Dhéré & Vegezzi (1916). The reaction, as has already been mentioned, is also given with acetone or glycol monomethyl ether, both of which are water-miscible solvents. But dioxan used in conjunction with NaOH, though freely miscible with water, failed to give dihydroxyl-protohaem. When dioxan was added to a solution of haematin either in 1% (w/v) Na_2CO_3 or in 0·1N-NaOH in the presence of $Na_2S_2O_4$, the solution became a clear cherry red and its two-banded absorption spectrum closely resembled that of caffeine-haem (Keilin, 1943). However, dioxan greatly sharpened the absorption spectrum of CO-protohaem both in aqueous and ethanolic solution, shifting the absorption bands a little towards the blue end of the spectrum, in contrast to the action of caffeine on CO-protohaem.

Reactions of haems other than protohaem with sodium hydroxide

Whereas protohaem requires ethanol or a similar solvent for the formation of dihydroxyl-protohaem, other haems such as deutero-, haemato- and urohaem form their corresponding dihydroxyl compounds without the addition of any organic solvent (Fig. 3). None of these haems, however, would give dihydroxyl compounds with Na_2CO_3 instead of NaOH. The formation of dihydroxyl-haematohaem and dihydroxyl-urohaem with increasing concentrations of NaOH were followed spectrophotometrically as described for dihydroxyl-protohaem. The minimum concentration of NaOH required for the formation of dihydroxyl-haem compounds was found to be 1·33N for protohaem in the presence of 50% ethanol and only 1·0N for haemato- and urohaem in the complete absence of ethanol. The presence of ethanol, nevertheless, slightly sharpens and shifts the absorption bands of dihydroxyl-haematohaem towards the red end of the spectrum. It affects similarly, but to a lesser degree, those of dihydroxyl-urohaem.

Stoicheiometric relationships

In order to determine the number of hydroxyl groups combined with each molecule of haem to give the hydroxyl compound, the haem was titrated with NaOH using the double wedge-trough method described by Keilin & Hartree (1946). Haematohaem was selected for this experiment, since it does not require ethanol for the formation of its hydroxyl compound and is more stable to reoxidation by air than urohaem.

3 ml. of a $6{\cdot}12 \times 10^{-4}$M-solution of haematohaematin in 1% Na_2CO_3 were diluted with 1% Na_2CO_3 to 25 ml. for one compartment of the double wedge trough, and with 1·5M-NaOH for the other compartment, so that the haematin concentration was $7{\cdot}35 \times 10^{-5}$ M in each compartment of the

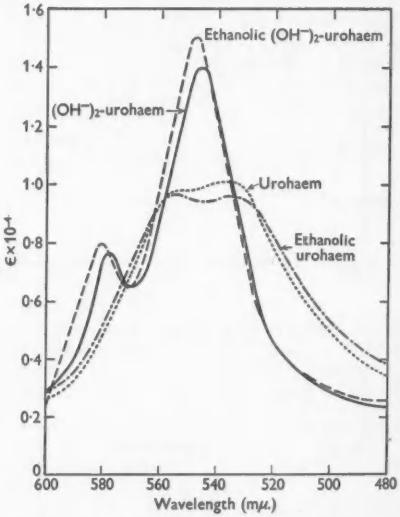


Fig. 3. Absorption bands in the visible region of the spectrum of $(OH^-)_2$-urohaem, urohaem, ethanolic urohaem and ethanolic $(OH^-)_2$-urohaem. The two latter solutions contain 50% v/v ethanol. (Urohaem = $1{\cdot}263 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.)

trough. The volume (v) of $6{\cdot}12 \times 10^{-4}$M-haematohaematin for the required optical depth in the cup was calculated as follows: $v = \pi r^2(c_1 l/c_2)$, where c_1 and c_2 = haematohaematin concentrations in trough and cup, respectively, l = light path in trough (2·1 cm.) and r = radius of cup. All the solutions were reduced with $Na_2S_2O_4$ and the haematohaem in the cup was matched against the broad end of the haematohaem compartment of the trough which was at zero position, the light intensities of the two spectra having been carefully equalized. The haem in the cup was then titrated with N-NaOH and the spectrum of the solution was matched, after each addition of NaOH, against the optical mixture of haematohaem and its hydroxyl compound in the two compartments of the trough. The concentrations

Table 1. *The relation between the sodium hydroxide concentration and percentage formation of dihydroxyl-haematohaem as determined experimentally and as calculated from the results by the method of least squares*

Total NaOH added (ml.)	NaOH concentration in solution		Percentage formation of compound	Percentage free haem	$\log\{x/(a-x)\}$	
	M	log M	(x)	($a-x$)	Determined experimentally	Calculated
0·05	0·238	−0·6231	16	84	−0·7201	−0·718
0·10	0·384	−0·4157	36	64	−0·2495	−0·286
0·25	0·612	−0·2132	56	44	+0·0864	+0·135
0·35	0·688	−0·1624	65	35	+0·2669	+0·240
0·45	0·740	−0·1308	62	38	+0·2253	+0·306
0·55	0·775	−0·1107	70	30	+0·3674	+0·348
0·70	0·814	−0·0894	72	28	+0·4099	+0·392
0·80	0·825	−0·0835	73	27	+0·4314	+0·409

of haematohaematin in the cup and trough were specially selected so that the spectrum of the haematohaem was clearly visible, while that of the fully formed hydroxyl compound in the trough

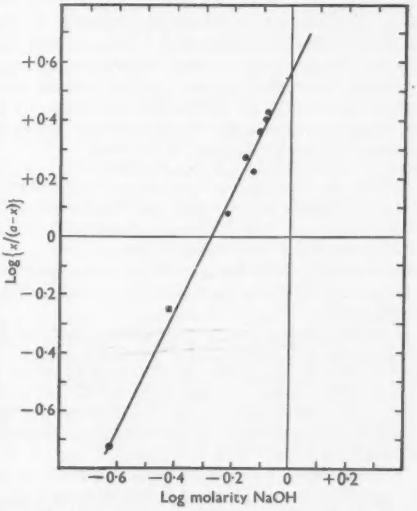


Fig. 4. Relationship between log molarity of NaOH and $\log\{x/(a-x)\}$, where x = percentage formation of $(OH^-)_2$-haematohaem and $a-x$ = percentage of free haematohaem. Experimental results are represented by dots. Results calculated from the equation

$$\log\{x/(a-x)\} = n \log (OH^-) - \log K,$$

where $n = 2{\cdot}08$ and $K = 0{\cdot}264$, are represented by the straight line.

(containing excess NaOH) was not too intense to prevent matching with the solution in the cup. It was thus possible to determine the percentage formation of the hydroxyl compound at different concentrations of NaOH and the results are given in Table 1.

If the reaction is represented by the following equation:

$$\text{Haem} + n(OH^-) \rightleftharpoons \text{Haem}(OH^-)_n,$$

then from the law of mass action

$$\frac{[\text{Haem}\,(OH^-)_n]}{[\text{Haem}]\,[OH^-]^n} = K.$$

If x represents the amount of the hydroxyl compound formed and a represents the total haem present, then

$$\frac{x}{(a-x)\,[OH^-]^n} = K,$$

whence $\log\{x/(a-x)\} = n \log [OH^-] - \log K$.

By applying the method of least squares to the values obtained for $\log\{x/(a-x)\}$ and $\log [OH^-]$, it was found that $n = 2{\cdot}08$ and $\log [OH^-] = -0{\cdot}278$ at 50 % formation of the hydroxyl compound while $k = 0{\cdot}264$, since under these conditions $\log K = n \log [OH^-]$. (This value for k does not take into account the very slight depression of $[OH^-]$ due to the presence of $Na_2S_2O_4$, nor the activity coefficient of NaOH.) On substituting these values for n and K in the above equation, the theoretical values of $\log\{x/(a-x)\}$ for different concentrations of NaOH can be calculated. These calculated values, together with those obtained experimentally, are shown in Fig. 4. This experiment shows conclusively that in the hydroxyl-haemato-haem compound the haem Fe combines with two hydroxyl groups, and since the corresponding compounds obtained with other haems have similar properties, all these compounds may be described under the name of dihydroxyl-haems.

Reactions of dihydroxyl-haems with carbon monoxide

When a current of CO is passed through a solution of dihydroxyl-protohaem, the latter assumes a bright cherry-red colour and its absorption bands are shifted to the following positions: α, 563; β, 532 and γ, 410 mμ. This spectrum corresponds to that of a solution of ethanolic CO-protohaem (Fig. 5). Similarly, the CO derivatives of dihydroxyl-haemato-haem and dihydroxyl-urohaem in aqueous solution are indistinguishable from their respective aqueous solutions of CO-haems.

At this point it may be interesting to recall Dhéré & Vegezzi's (1916) experiments on the

reduction of acid and alkaline ethanolic solutions of protohaematin in open and sealed tubes. They invariably found that in sealed tubes the absorption spectra of the solutions were very different from those in open tubes and, although the latter contained an excess of reducer, they believed at first that complete reduction could only be obtained in sealed tubes. However, later Dhéré (1927) recognized that the difference was not due to the more complete reduction of the solutions in sealed tubes but to the formation within them of CO-protohaem, the CO having been produced by incomplete combustion of ethanol during the process of sealing the tubes.

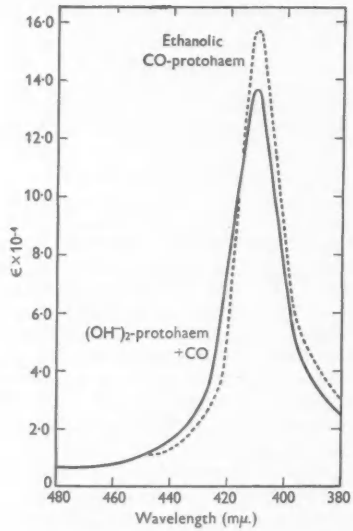


Fig. 5. Absorption spectra showing γ-bands of $(OH^-)_2$-protohaem + CO and of CO-protohaem containing 50% v/v ethanol. (Protohaem = $1{\cdot}85 \times 10^{-5}$ M, $l = 0{\cdot}5$ cm.)

The magnetic susceptibility of dihydroxyl-urohaem

The method of Gouy (1889) was adapted for use with an 'Alcomax' permanent magnet giving a uniform field of 10,600 gauss within a gap of 6 mm. The magnet was mounted on a jack so that it could be moved vertically with precision and without any lateral displacement of the magnet. The magnet and jack were enclosed in a glass case and kept at constant temperature (20·5°). A narrow glass tube (10 cm. long, 2 mm. internal diam. and 0·3 ml. capacity), fitted with glass suspending hooks at the open end and closed with a rubber cap, was hung on a platinum wire from the pan of the balance and weighed with its lower end between the pole pieces of the magnet. The magnet was then lowered about 25 cm. by means of the jack, and the tube, now in a negligible magnetic field, was weighed again. The increase in weight, Δw, was then similarly determined (*a*) with the tube full of distilled water, (*b*) with the tube filled with the haem solution under investigation, and (*c*) with the tube filled with the solvent (i.e. all the constituents except the haemin). These changes in weight were each corrected for the change in weight of the empty tube Δw, and are represented as Δw_{H_2O}, $\Delta w_{soln.}$ and $\Delta w_{solv.}$ respectively.

The mass susceptibility χ of the solution was calculated according to the following formula, derived from the equation given by Trew & Watkins (1933):

$$10^6 \chi_{soln.} = \left(10^6 \chi_{H_2O} - \frac{K_{air}}{d_{H_2O}}\right) \frac{\Delta w_{soln.} \cdot d_{H_2O}}{\Delta w_{H_2O} \cdot d_{soln.}} + \frac{K_{air}}{d_{soln.}},$$

where χ_{H_2O}, the mass susceptibility of water $= -0{\cdot}72 \times 10^{-6}$; K_{air}, the volume susceptibility of air $= +0{\cdot}0294 \times 10^{-6}$; and d = specific gravity.

The value of $10^6 \chi_{solv.}$ was similarly calculated and the mass susceptibility of the solute (haem) was obtained from:

$$\chi_{soln.} = c\chi_{solute} + (1 - c)\chi_{solv.}$$

where c = wt. of dissolved haemin/wt. of solution.

The magnetic moment μ_B (in Bohr magnetons) is then given by

$$\mu_B = 2{\cdot}84\sqrt{(\chi MT)},$$

where χ = mass susceptibility of haem compound, M = mol.wt. of haemin, T = absolute temperature.

Urohaem was selected for these experiments instead of protohaem in view of its greater solubility and the ease in bringing the reaction to completion. The magnetic susceptibilities of protohaematin, dicyan-protohaem and dicyan-urohaem were also determined by the same method. This was done in order: (1) to test the accuracy of the procedure, (2) to test the suitability of the urohaem preparation for such measurements, and (3) to provide the data necessary for comparison with those obtained for dihydroxyl-urohaem.

The solutions were made up as follows:

Protohaematin. Protohaemin (50 mg.) was dissolved in 5 ml. 0·2 N-NaOH containing about 30% sucrose (Pauling & Coryell, 1936).

Dicyan-protohaem. Protohaemin (60 mg.) was dissolved in about 6 ml. of the above sucrose-NaOH solution. KCN (300 mg.) and $Na_2S_2O_4$ (100 mg.) were then added and the volume of the solution was made up to 10 ml. with sucrose-NaOH. (This order was adopted to avoid the gelatinous precipitate which tended to form if the solid haemin was added last.)

Dicyan-urohaem. Urohaemin (20 mg.), KCN (30 mg.) and $Na_2S_2O_4$ (20 mg.) were dissolved in that order in 1 ml. 1% Na_2CO_3 contained in a small, stoppered, graduated bottle with a narrow neck to minimize the air space above the fluid. The solution was transferred by means of a Pasteur pipette to the magnetic susceptibility tube which was completely filled and closed with the rubber cap. These precautions to exclude contact with air were necessary on account of the extreme ease with which the reduced urohaem compounds are oxidized in air.

Dihydroxyl-urohaem. Urohaemin (20·7 mg.) and $Na_2S_2O_4$ (20·3 mg.) were dissolved in 1 ml. M-NaOH, observing the precautions described above.

The results of these experiments, which were carried out at 20·5° and which are summarized in Table 2, show that the Fe in dihydroxyl-urohaem has a much higher magnetic moment than the Fe in dicyan-urohaem. The values obtained for protohaematin and dicyan-protohaem are in agreement with those given by Pauling & Coryell (1936) for

Table 2. *The values for Δw and specific gravity (d) of water, solvent and solution, and the molar susceptibility χ_m and magnetic moment μ_B (in Bohr magnetons) of protohaematin, dicyan-protohaem, dicyan-urohaem and dihydroxyl-urohaem*

(Approx. 30% sucrose was used only in the case of protohaematin and dicyan-protohaem.)

Compound	Solvent: $Na_2S_2O_4$ (mg./ml.)	Solvent: KCN (mg./ml.)	Solvent: Alkali and sucrose	Δw_{H_2O}	$d^{20\cdot5}_{H_2O}$	$\Delta w_{solv.}$	$d^{20\cdot5}_{solv.}$	Δw_{soln}	$d^{20\cdot5}_{soln.}$	$10^6\chi_m$	μ_B
Proto-haematin	—	—	30% sucrose, 0·2 N-NaOH	4·142	0·998	4·2498	1·1193	3·048	1·1223	13,830	5·722
Dicyan-protohaem	10	30	30% sucrose, 0·2 N-NaOH	4·022	0·998	4·0724	1·1415	4·137	1·1455	0	0
Dicyan-urohaem	20	50	1% Na_2CO_3	2·533	0·998	2·740	1·0412	2·631	1·0445	909	1·46
Dihydroxyl-urohaem	20·1	—	N-NaOH	2·44	0·998	2·70	1·056	1·673	1·0645	13,585	5·672

both compounds and by Haurowitz & Kittel (1933) for protohaematin. The relatively high values obtained for both the urohaem compounds are probably due to some degree of oxidation that occurred in spite of the precautions taken to avoid it.

DISCUSSION

The solubility of a haemin derivative depends very largely on that of its parent porphyrin and the porphyrins differ greatly in the nature of their side chains. Carboxyl groups in general confer solubility on the molecule so that uroporphyrin, with its eight carboxyl groups, is the most soluble of the porphyrins. Haematoporphyrin, with —CH(OH).CH_3 groups in positions 2 and 4, is more soluble than proto-porphyrin which has vinyl groups (—CH=CH_2) in these positions. Haemin derivatives also differ in solubility according to the valency of their iron. Thus the ferrous compounds such as haem and haemochromogen are less soluble than their ferric forms, haematin and parahaematin. It is also interesting to note that CO-haem is more soluble than free haem.

It is well known that the solution or dispersion of molecular aggregates of a pigment is accompanied by an intensification of its absorption bands and by a more or less marked shift of these bands towards the blue end of the spectrum. In fact, the addition of ethanol intensifies the absorption bands of haems, and this intensification, which varies inversely with the solubility of the haem in dilute aqueous alkali, is therefore more marked for proto- than for haematohaem and is very feeble in the case of urohaem.

The conditions required for the formation of dihydroxyl-haem compounds are (*a*) a high concentration of alkali (1–1·33 N-NaOH), and (*b*) a certain degree of solubility of the haem. In the case of protohaem, which is relatively insoluble, the required degree of solubility can only be obtained by the addition of an appreciable amount of ethanol (up to 50%) or of another water-miscible solvent such as acetone or glycol monomethyl ether. On the other hand, ethanol is not required for the formation of dihydroxyl-urohaem and, when added, hardly affects the spectrum, as urohaem itself is very soluble in very dilute alkali. In this respect haematohaem occupies an intermediate position between proto- and uro-haem. Although dihydroxyl-haematohaem, with its characteristic absorption spectrum, may be formed in the complete absence of ethanol, the addition of the latter intensifies its bands and

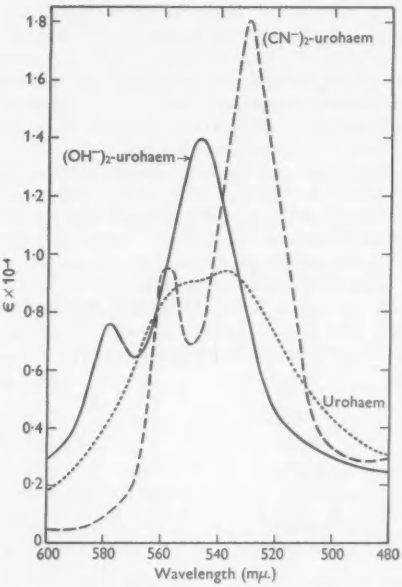
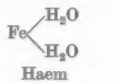


Fig. 6. Absorption bands in the visible region of the spectrum of $(OH^-)_2$-urohaem, urohaem (urohaem = $1{\cdot}263 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.) and $(CN^-)_2$-urohaem (urohaem = $6{\cdot}61 \times 10^{-5}$ M, $l = 0{\cdot}25$ cm.).

shifts them slightly towards the red end of the spectrum. This change is brought about by a simultaneous increase in the solubility of the haematohaem in presence of ethanol and a decrease in that of its dihydroxyl compound.

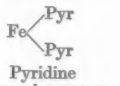
Information as to the nature of the hydroxyl-haem compound was obtained in two ways: (1) Directly, by titrating the haem with NaOH and determining its dissociation constant. This showed that two hydroxyl groups were combined with each atom of haem Fe (see Table 1 and Fig. 4). (2) Indirectly, by comparing its properties with those of other haem derivatives such as haem, caffeine-haem, haemochromogens, monocyan- and dicyan-haem and monocarbylamine- and dicarbylamine-haem. The absorption spectra of dihydroxyl-haems and the corresponding dicyan-haems bear a striking resemblance to each other (Figs. 1 and 6). This resemblance, which can be seen in the visible and the violet regions of the spectrum, applies to the general pattern of the absorption spectrum as reflected in the relative intensities of the bands. The fact that the general spectral pattern common to dihydroxyl-haems, dicyan-haems and dicarbylamine-haems differs so markedly from those of all other haematin derivatives suggests that these compounds have similar structures.

Spectroscopic and potentiometric investigations (see Anson & Mirsky, 1925; Hill, 1929; Davies, 1940; Shack & Clark, 1947) suggest that the structures of different haem (ferrous) compounds can be represented as follows (in these schematic representations the lines joining the haem Fe to the reacting groups do not indicate the nature of the valency bonds; the four valencies directed towards the pyrrole nitrogen atoms are omitted):



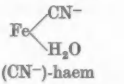
Haem

CO-haem



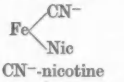
Pyridine haemochromogen

CO-pyridine haemochromogen



(CN^-)-haem

$(CN^-)_2$-haem



CN^--nicotine haemochromogen

CO-CN^--haem

By analogy with dicyan-haem, the structure of dihydroxyl-haem may therefore be represented as

The study of Dhéré & Vegezzi's (1916) compound, apart from its structural relationship with different haem derivatives, is important in two other respects. (1) Optical and magnetic studies on different haematin compounds have suggested the existence of a certain empirical relationship between the pattern of their absorption spectra and the type of iron bonding in each compound as determined by magnetic susceptibility measurements (Theorell, 1942; Hartree, 1947). In this respect the dihydroxyl-urohaem presents a striking exception. Although the absorption spectra of dihydroxyl-urohaem and dicyan-urohaem are of the same pattern, the magnetic measurements show fundamental structural differences. While the Fe atom in dihydroxyl-urohaem is linked by ionic bonds, with the result that the compound shows the high magnetic moment characteristic of free haems, the bonds in dicyan-urohaem are essentially covalent and the low magnetic moment brings it into line with the diamagnetic haemochromogens. In view of the strict analogies existing between the corresponding compounds of different haems it is reasonable to assume that the results obtained for urohaem may be taken to apply to dihydroxyl compounds of other haems. (2) Strong alkalis, with or without ethanol, are often used in studying the reactions between haems and different nitrogenous substances. However, unless one is familiar with the absorption spectrum of dihydroxyl-haem and aware of its possible appearance during the course of the reaction, the main absorption band of this compound can easily be mistaken for that of a partly developed haemochromogen, since its strong β-band can be seen where the α-band of the haemochromogen is expected to appear. This aspect of the problem will be discussed more fully elsewhere.

SUMMARY

1. It is shown that Dhéré & Vegezzi's (1916) 'alkaline haemochromogen' is a compound in which the haem iron is combined with two hydroxyl groups and it is therefore named dihydroxyl-haem.

2. For the full formation of dihydroxyl-protohaem 1·33 N-NaOH and 50% (v/v) ethanol are required. Ethanol, however, can be replaced by other water-miscible organic solvents such as acetone and glycol monomethyl ether, but not by dioxan.

3. In the case of haematohaem and urohaem the corresponding dihydroxyl compounds can be obtained in complete absence of an organic solvent, although the absorption spectrum of dihydroxyl-haematohaem is reinforced by ethanol.

4. The dissociation constant K for dihydroxyl-haematohaem is 0·264 at 20°.

5. The absorption spectrum of dihydroxyl-haem differs markedly from those of haem, caffeine-haem,

haemochromogens and monocyan-haem, but shows a striking resemblance to those of dicyan-haem and dicarbylamine-haem.

6. The bearing of the similarity of the absorption spectra of dicarbylamine-haem, dicyan-haem and dihydroxyl-haem on the structure of this compound is discussed.

7. Dihydroxyl-haem, unlike dicyan-haem, is paramagnetic, and when treated with carbon monoxide gives CO-haem.

8. The knowledge of the absorption spectrum and other properties of the dihydroxyl-haem compound is of special importance for the study of the reactions between haems and nitrogenous compounds.

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The Semi-micro Estimation of Lactose Alone and in the Presence of Other Sugars

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In the course of studies on the intermediary metabolism of the mammary gland, and in particular the investigation of lactose formation in incubated tissues, it became necessary to devise improved methods for estimating this sugar in the presence of glucose and glycogen. The methods hitherto available for the estimation of small amounts of lactose, alone or in the presence of other sugars, are laborious and unsatisfactory. Those normally used for estimating lactose in milk suffer from a lack of specificity which makes them valueless in any investigation where mixtures of sugars and related carbohydrates are likely to be found, while those designed for more general use rely on the complementary techniques of yeast fermentation, for removing fermentable sugars, and the use either of reduction methods or of a lactose-fermenting yeast to give the lactose equivalent of the residual fermented solutions (Grant, 1935; Scott & West, 1936). The application of paper chromatography to the separation and characterization of sugars in mixtures (Partridge, 1946, 1948), and the adaptation of this method to the estimation of sugars (Flood, Hurst & Jones, 1947; Hawthorne, 1947), are advances of great value, but the techniques are time-consuming and, for other reasons, frequently unsuited for routine work (see Westall, 1948).

In the hope of obtaining a method which would be more convenient in practice and yet have greater specificity than those ultimately dependent on estimations of reducing power, the colour reaction given by lactose with methylamine in alkaline solution, first observed by Fearon (1942) and later used in qualitative studies for detecting lactosuria in pregnancy and the early puerperium (Harwood, 1946; Archer & Haram, 1948), has been developed into a quantitative method. Fearon found that the

reaction was not given by other sugars (except maltose), or by many other substances, including lactic and pyruvic acids, normally associated with carbohydrate metabolism. Since maltose can readily be removed by fermentation the reaction clearly offered the possibility of a specific estimation for lactose which might satisfy an urgent need in studies on the metabolism of the mammary gland.

EXPERIMENTAL

Reagents

Lactose. Pure bacteriological (Kerfoot).

Methylamine hydrochloride. Prepared from 40% methylamine in water (L. Light and Co., Ltd.) by neutralizing with HCl, evaporating the solution to dryness on a water bath, extracting with and recrystallizing from ethanol.

Zinc hydroxide. Prepared from zinc acetate by the method of Letonoff (1934).

Estimation of lactose alone in aqueous solution

The solution (8 ml. containing 4–16 mg. lactose) is pipetted into a boiling tube (6 × 1½ in.) and treated with 6 drops (0·32 ml.) 10% (w/v) methylamine-HCl solution. The tube is loosely stoppered with a glass cap and the mixture heated in a boiling water bath. After heating for 7 min. and 5 sec., 0·4 ml. of 4·4 N-NaOH are added, and 10 sec. later the tube is removed from the bath and the contents mixed and allowed to cool in air. The red colour develops in the stoppered tube during the course of the next 2 min., and is measured in a Spekker photoelectric absorptiometer using a spectrum green filter (Ilford no. 604, 520 mμ.) in conjunction with heat-absorbing filters (Hilger H 503). The solutions are read against water in the second cell and with the drum setting initially at unity.

To ensure maximum colour development the estimations are carried out with strict adherence to a time schedule. They may be done conveniently in an overlapping series, when 6 or 7 may be completed in 1 hr. The full time scheme is as follows for the first two members, *A* and *B*, of a series:

0 sec.	Add 6 drops 10% methylamine-HCl to *A*.
15 sec.	Place *A* in boiling water bath and insert stopper.
7 min. 20 sec.	Add 0·4 ml. 4·4 N-NaOH to *A*.
7 min. 30 sec.	Remove *A* from bath.
9 min. 0 sec.	Add 6 drops 10% methylamine-HCl to *B*.
9 min. 15 sec.	Place *B* in boiling water bath and insert stopper.
9 min. 30 sec.	Filter *A* directly into absorptiometer cell. (This is unnecessary in the case of simple aqueous solutions, but essential when salt solutions containing Ca^{++} or Mg^{++} are used, or when the estimations are on biological extracts).
10 min. 45 sec.	Measure *A* in absorptiometer.
16 min. 20 sec.	Add 0·4 ml. 4·4 N-NaOH to *B*, and continue as outlined above.

Under these conditions the calibration curve for standard lactose solutions is linear over the range 4–16 mg. (0·05–0·2%); below this range the increase in the colour with increase in concentration is less pronounced, and the method, although useful, is decidedly less accurate for solutions between 0·025 and 0·05%. (Fig. 1).

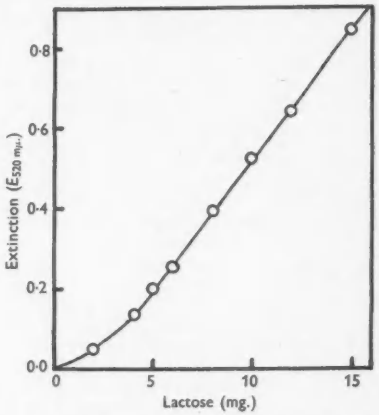

Fig. 1. Calibration curve for standard lactose solutions.

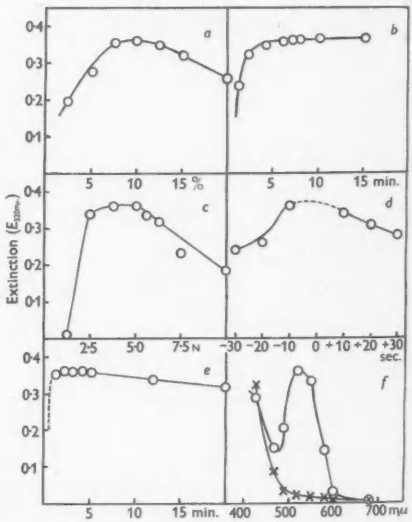

Fig. 2. Investigation of factors affecting the estimation of lactose (0·1% (w/v); cell 1 cm.); (*a*) strength of methylamine-HCl solution; (*b*) time of heating; (*c*) strength of NaOH; (*d*) time of addition of NaOH relative to removal from the water bath; (*e*) rate of development of colour after removal from the water bath; (*f*) absorption spectra of lactose compound O——O and glucose compound ×——×.

The results of an investigation into the optimum conditions of the reaction are given in Fig. 2. The effects of altering the strength of the methylamine-HCl solution (*a*),

the time of heating (*b*), strength of NaOH (*c*), time of addition of NaOH relative to the time of removal from the water bath (*d*), and the time of estimating after removal from the water bath (*e*) are shown, together with the visible absorption spectrum of the lactose compound (*f*). The first, third and fourth of these possible variants are clearly critical and must be strictly controlled. In the further work to be discussed in this paper 5 N-NaOH was used; from a reassessment of the method, however, it would seem preferable to use a slightly weaker solution and 4·4 N-NaOH is now recommended. The choice of the time for adding the NaOH relative to removing the tube from the water bath necessarily involves considerations of technical convenience, and although there are indications, both from experiment and

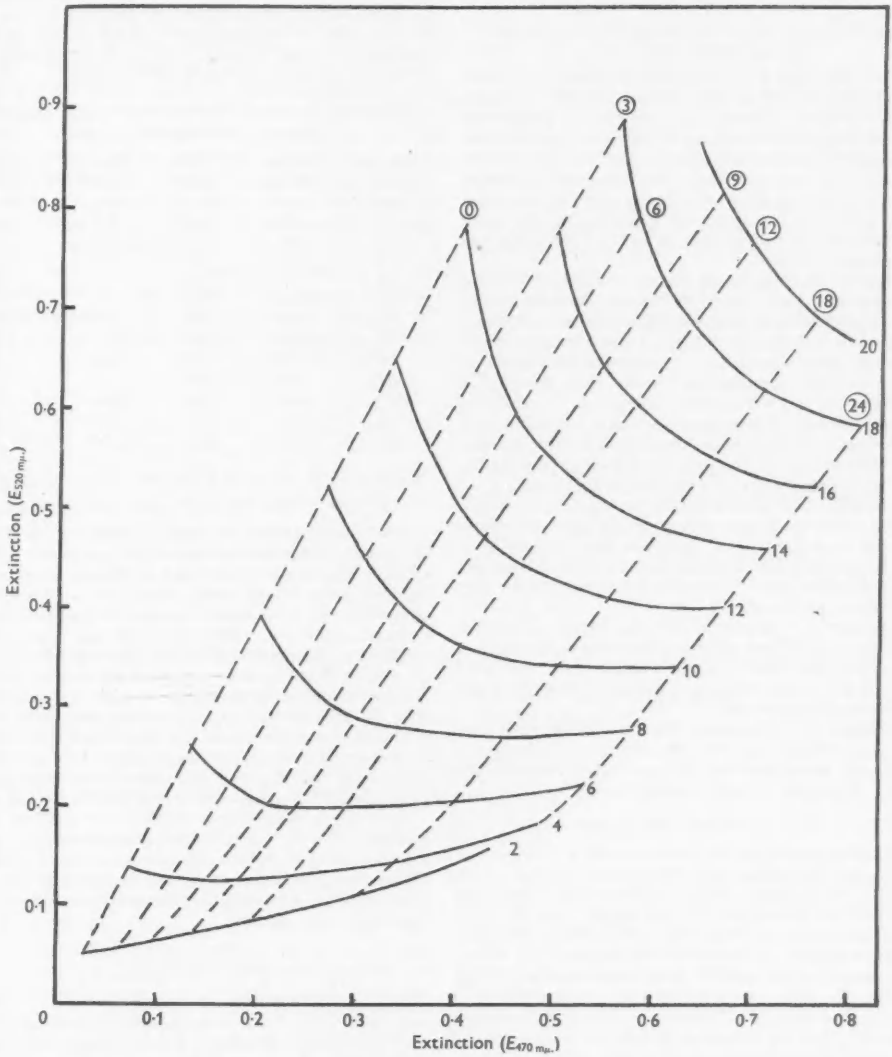

Fig. 3. Composite graph used in estimating lactose in the presence of glucose: ——, curves for constant lactose concentration, equivalent (mg. lactose) given in plain numbers; -----, curves for constant glucose concentration, equivalent (mg. glucose) given in 'circled' numbers.

from an interpolation of partial curves (*d*), that the extinction values would be slightly higher if the removal coincided with the addition of NaOH, the small gain in sensitivity would be more than offset by the manipulative inconvenience.

Estimation of lactose in the presence of glucose in aqueous solution

When estimated in the same way, glucose gives a yellow colour instead of red, and it is therefore possible to estimate lactose in the presence of glucose, without preliminary fermentation, by measuring the optical density at two wavelengths. The additional wavelength selected (470 mμ., Ilford no. 602, used in conjunction with heat-absorbing filters, Hilger H 503) gives appreciable, but suboptimal, absorption for glucose alone (see absorption spectrum, Fig. 2(*f*)), and coincides approximately with a point of relatively low absorption for lactose.

From the results given by a series of standard mixtures covering the range lactose 0–20 mg., glucose 0–24 mg., curves are drawn, by plotting the extinction at 520 mμ. against the extinction at 470 mμ., relating these values to constant lactose concentrations measured in the presence of varying glucose concentrations (Fig. 3). From these curves lactose values may be read directly for any pair of extinction values. The method introduces a source of subjective error in the need for interpolating values between the standard curves thus obtained. Interpolation is made on lines parallel to the transverse curves (broken lines in Fig. 3) connecting points of the same glucose content on the different lactose curves. These transverse curves clearly allow the simultaneous determination of glucose in the solutions by a similar process of interpolation, this time along lines parallel to the standard lactose curves. The following examples may be demonstrated on Fig. 3.

Example I. Extinction 520 mμ.=0·200; extinction 470 mμ.=0·240. These readings give a point on the intersect of the 6 mg. standard lactose curve and the 9 mg. transverse glucose curve, and indicate a solution containing 6 mg. lactose and 9 mg. glucose.

Example II. Extinction 520 mμ.=0·272; extinction 470 mμ.=0·238. These readings involve interpolation for both the lactose and the glucose values, and give the result, 7·2 mg. lactose and 5 mg. glucose.

Sensitivity of the method

For simple solutions of lactose the accuracy of the method in the most favourable range (0·05–0·2%) falls well within ±5%. In the presence of glucose the accuracy varies with the relative amounts of the two sugars, and the direct application of the method is not recommended, except for qualitative work, for solutions containing less than 0·05% lactose, or for any solution containing more than 0·15% glucose. In such cases preliminary fermentation should be carried out and the lactose determined alone in the fermented solution. It is our experience that in the range suggested here lactose can be estimated with an accuracy of ±5%; the glucose value is much less accurate (Table 1).

Estimation of lactose in the presence of glycogen

Solutions of glycogen give a strong opalescence when treated according to the standard method, and it is essential to remove glycogen before the estimation. This is done by precipitation with an equal volume of ethanol. After standing for 5 min. the solution is filtered and suitable samples of the ethanolic filtrate transferred to boiling tubes; the solutions are evaporated to dryness on a water bath, the residue dissolved as far as possible in 8 ml. distilled water and the estimation carried out as in the standard method. If salts are present it is necessary to adjust the pH of the solutions to between 7·0 and 7·4 before drying, in order to prevent decomposition or charring of the sugar.

Table 1. *Recoveries of lactose from lactose-glucose solutions of varying composition*

Lactose present (mg.)	Glucose present (mg.)	Lactose found (mg.)	Glucose found (mg.)	Lactose error (%)
1·8	0·6	1·6	0·9	−11
1·8	1·8	1·6	2·2	−11
1·8	6·0	1·6	6·0	−11
2·4	1·2	2·2	1·9	− 8
2·4	2·4	2·3	2·7	− 4
2·4	6·0	2·0	6·3	−17
3·0	12·0	2·9	11·2	− 3
3·0	15·0	3·0	13·7	0
6·0	9·0	6·0	7·0	0
6·0	24·0	6·0	20·0	0
18·0	6·0	16·8	2·2	− 7
18·0	12·0	17·6	8·5	− 2

Estimation of lactose in solutions containing soluble nitrogen (tissue 'incubates') and salt mixtures

Colour development is partially inhibited in tissue 'incubates' unless such solutions are previously deproteinized. The use of strongly acid or alkaline reagents for deproteinization would clearly complicate the subsequent estimation, and in the search for a neutral precipitant zinc hydroxide (Letonoff, 1934) was tried and found most satisfactory. It has the additional advantage that its use involves no alteration in the volume of the solutions treated and no diminution of the lactose content. Approximately 0·1 g. $Zn(OH)_2$ is added/30 ml. of solution, and the mixture is allowed to stand for 5 min. and then filtered. The filtrate is used for the estimation without further treatment. The addition of $Zn(OH)_2$ may be omitted when glycogen is present, since the ethanol also precipitates the protein.

Solutions pre-treated with $Zn(OH)_2$, or solutions containing Ca^{++} or Mg^{++} (e.g. Ringer-phosphate solution), give a precipitate of insoluble hydroxides when NaOH is added during the estimation. In such cases the solutions are filtered through Whatman no. 1 filters at 9 min. 30 sec. (see time scheme for method).

DISCUSSION

We have extended Fearon's (1942) observations on the specificity of the lactose-methylamine reaction and find that, in addition to maltose, cellobiose and certain lower dextrins give a similar colour. The dextrins are only slowly fermented by brewer's yeast and are thus readily distinguishable from maltose which is rapidly fermented. A limited investigation of their reduction values before and after acid hydrolysis, using the method of Somogyi (1945), gave ratios of approximately 1/5. It seems probable, therefore, that the specificity of the

reaction extends to disaccharides and lower polysaccharides containing the 1:4 glycosidic linkage, irrespective of their stereochemical relationships. In agreement with this, sucrose (1:2) and melibiose (1:6) fail to give the reaction, as do the trisaccharides raffinose and melezitose.

The method seems to offer scope for further application to mixtures of lactose with other sugars, either by direct measurement—when the contaminating sugar gives no colour (raffinose)—or by measurement at two selected wavelengths, when interfering colours are produced (galactose). Estimations in the presence of maltose or lower dextrins may be made after preliminary fermentation with brewer's yeast, though the slow and variable fermentation rates of the latter, presumably to be associated with differences in the degree of polymerization of the dextrins capable of giving a red colour, may present special difficulties. When cellobiose is present an approximation to the correct lactose concentration can be made by first acting upon the solution with emulsin, which hydrolyses β-glycosides much more rapidly than lactose.

SUMMARY

1. A method is described for the semi-micro estimation of lactose; modifications are given for its application to solutions containing lactose in the presence of glucose and glycogen, and in tissue extracts, and more general recommendations made for its use when other contaminating sugars are present.

2. The method is most sensitive for lactose concentrations in the range 0·05–0·2 %.

We wish to express our thanks to Prof. D. C. Harrison for his interest in the progress of this work and to Dr Q. H. Gibson for valuable suggestions with regard to the graphical presentation.

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Displacement Chromatography on Synthetic Ion-exchange Resins

4. THE ISOLATION OF GLUCOSAMINE AND HISTIDINE FROM A PROTEIN HYDROLYSATE

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In Part 3 of this group of papers (Partridge, 1949) the fractionation of the hydrochloric acid hydrolysis product of commercial egg albumin was described. A displacement chromatogram, using a column packed with 'Zeo-Karb 215' showed the presence of seven discrete bands each of which contained simple mixtures of amino-acids. These were identified by carrying out partition chromatograms on filter paper by the method of Consden, Gordon & Martin (1944). Band VI contained histidine together with an unknown basic substance. This substance has now been identified as glucosamine and both glucosamine and histidine have been isolated from the amino-acid mixture in a pure condition. The amount of glucosamine in the hydrolysis product is very small and its origin is probably the ovomucoid contained in white of egg. Histidine is also present in small amount, the content of this amino-acid in crystalline ovalbumin being about 2·4 % (Chibnall, 1945).

The following is the order in which the more basic amino-acids appear in the effluent from a column of 'Zeo-Karb 215' when ammonia solution is used as the displacement developer: leucine, histidine, glucosamine, lysine, ammonia. The affinity of glucosamine for the resin is not much greater than that of histidine, and separation is only partial with columns of normal length. It has been pointed out (Partridge & Westall, 1949; Davies, 1949) that when the displacement developer is a free base, the order of displacement reflects the ability of the stronger bases to control the pH of the aqueous phase and thus to depress the cationic form of the weaker bases.

Davies (1949) shows that the controlling equilibrium in the case of the amino-acids may be represented by

$$A^+ \rightleftharpoons A^{\pm} + H^+. \quad (1)$$

In addition, there may be two further factors affecting the adsorption of an amino-acid by the resin: (*a*) van der Waals forces between the resin and the cation A^+ or the neutral molecule $A^{\pm}$, and (*b*) the affinity of the cation for the resin will be greater the higher its valency.

It is clear, therefore, that by altering the environment of the ions in the ambient phase it would be possible to exaggerate the effect of one of these mechanisms at the expense of the others and thus to produce conditions in which the order of displacement is altered. Thus, by using a dilute solution of a salt as displacement developer the pH of the ambient fluid would be lowered and equation (1) would lie entirely to the left (in so far as univalent cations are concerned). This has been made use of to secure the separation of glucosamine from histidine. When a solution of a salt flows through a column containing an adsorbed mixture of amino-acids, the cations of the salt are adsorbed and the anions are released together with the cations of the displaced amino-acids. The high acidity thus set up results in the mechanism depending upon equation (1) becoming inoperative and the mutual displacement of the amino-acids is thus controlled by mechanisms (*a*) and (*b*) only. In this case, as would be expected, the bivalent cations are strongly adsorbed and the observed order of displacement where 0·1 M-sodium chloride is used as the displacement developer is as follows: glucosamine, Na^+, leucine, histidine, lysine. The affinities of Na^+ and the leucine cation are very similar, but with long columns the Na^+ band tends to overtake that due to leucine. Lysine and histidine are much more strongly adsorbed than Na^+, and the sodium band overruns these two amino-acids and shows little tendency to cause their migration down the column by elution.

METHODS

Comparison of the effects of sodium chloride and sodium hydroxide solutions as displacement developers

The behaviour of the two displacement developers was compared directly by setting up two small identical columns and analysing with each the same sample mixture of bases.

The columns each contained 2 g. of air-dried 'Zeo-Karb 215' (80–100 mesh/in.; height of column 6·1 cm.). Before use the columns were treated alternately several times with 2 N-HCl and 2 N-NaOH. In both cases the mixture of solutes run through the column (in its hydrogen form) was: histidine, 0·1 M, 10 ml.; glucosamine hydrochloride, 0·1 M, 10 ml.; glycine, 0·1 M, 10 ml.

Column (1) was developed by displacement with 0·1 N-NaOH applied at a rate of 1 ml./min. The effluent was collected in 3 ml. fractions, 34 fractions being collected in all. A filter-paper chromatogram was set up using a drop of solution from each fraction and the results are shown diagrammatically in Fig. 1*a*.

Column (2) was developed by passing at first a solution of NaCl (0·05 M) at a rate of 2 ml./min. A portion of the effluent (45 ml.) was allowed to pass before commencing to collect fractions. After 34 fractions (each of 3 ml.) had been collected the reservoir containing NaCl solution was replaced by one containing 0·1 N–NaOH and the experiment was continued until a further 26 fractions had been taken. The composition of the effluent was analysed by paper chromatography as before and the results are shown in Fig. 1*b*.

The effluent solution at first contained HCl arising by removal of the cations from the NaCl solution, but when the column became saturated with cations the hydrogen-ion concentration of the effluent fell rapidly and hydrochlorides of glycine and glucosamine appeared. Between fractions 10 and 14 (Fig. 1*b*) glucosamine and glycine hydrochloride were replaced by NaCl and the free acidity of the effluent fell to less than 0·001 N. On replacing the reservoir by one containing NaOH the residual NaCl solution was rapidly displaced and salt-free water flowed from the column until the appearance of the histidine band.

In order to collect data for the design of a suitable experimental procedure for the isolation of the components of the histidine-glucosamine band obtained in the primary separation, the experiment described above was repeated several times with small variations in the procedure. The results of these experiments may be summarized as follows.

(*a*) *Replacement of glycine by leucine.* The experiment illustrated in Fig. 1*b* was repeated with leucine in place of glycine. The chromatogram showed a good separation between glucosamine and leucine, the leucine front almost coinciding with that due to sodium chloride. The leucine band had a very diffuse rear boundary, showing that, under the conditions of the experiment, the affinities of the leucine and sodium ions for the resin were similar. A small part of the leucine was not eluted from the column by the volume of sodium chloride solution applied, and this appeared as a sharp but narrow band in front of the histidine band when the histidine was displaced by sodium hydroxide solution.

(*b*) *Effect of increasing the concentration of sodium chloride.* The sodium chloride concentration was increased to 0·1 M and the chromatogram compared with that shown in Fig. 1*b*. The result showed that while the glucosamine band was relatively shorter, and the glucosamine content of the effluent correspondingly higher, the leading edge of the band was very diffuse. This effect was probably due to the tendency of glucosamine to be eluted by the higher concentration of hydrochloric acid present.

(*c*) *Effect of replacing the sodium hydroxide solution by ammonia* (0·1 N). The resulting chromatogram showed an improvement in the sharpness of the histidine band. Since the use of sodium hydroxide

leads to difficulties where larger columns are employed, dilute ammonia solution was adopted for all further experiments.

(*d*) *Effect of increasing the column length.* The experiment was carried out using glucosamine hydrochloride, leucine and histidine as the experimental mixture. The increase in column length was about 25% and the chromatogram showed an improvement in the separation of glucosamine and leucine. It was considered that the extra column length allowed the sodium band to overtake that due to leucine, with

grams carried out on the mixed solution showed this to contain histidine and the 'unidentified substance', together with smaller quantities of leucine, lysine and cystine.

Identification of glucosamine

The unidentified substance was observed to have R_F values in a number of different solvents close to those given by glucosamine. Since the amino-sugar gives a good colour reaction with ninhydrin and is known to occur in the ovomucoid of white of egg, the

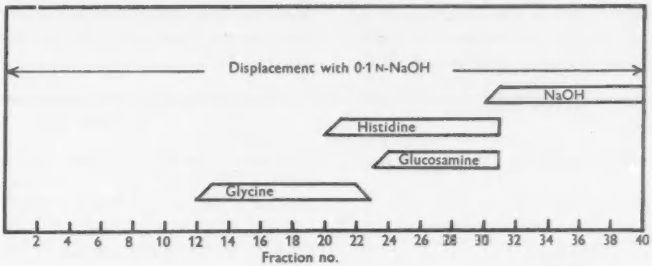
Displacement with 0·1 N-NaOH
NaOH
Histidine
Glucosamine
Glycine
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Fraction no.

Fig. 1*a*.

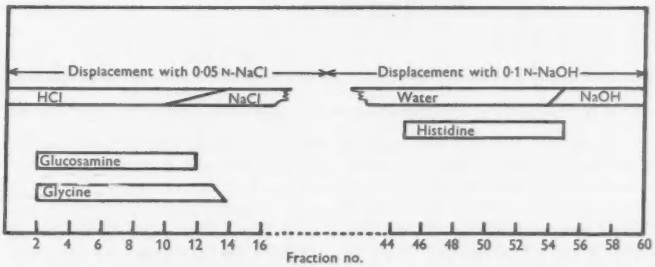

Fig. 1*b*.

Fig. 1. *a*, Composition of the effluent from a column of 'Zeo-Karb 215' on displacing a mixture of glycine, glucosamine, and histidine with 0·1 N-NaOH. *b*, Composition of the effluent obtained by displacing the amino-acid mixture first with 0·05 N-NaCl followed by 0·1 N-NaOH.

the result that a narrow zone of sodium chloride was interpolated between the glucosamine and the mixed sodium chloride-leucine bands.

Primary fractionation of the protein hydrolysate

A sample of commercial egg albumin (moisture 13·8%, ash 3·4%; 40 g.) was hydrolysed with N-hydrochloric acid. It was then treated with charcoal and fractionated on a column of 'Zeo-Karb 215' exactly as described in Part 3 of this series (Partridge, 1949). Using the filter-paper chromatogram as a guide, three fractions of the effluent (each of 47 ml.) were combined together so as to comprise the whole of the histidine band. Further filter-paper chromato-

identity of the two substances was probable. Like glucosamine, the unknown substance was shown to yield brown spots on chromatograms sprayed with ammoniacal silver nitrate and cherry-red spots with the Elson & Morgan (1933) reagents (cf. Partridge, 1948). Further chromatograms were carried out in phenol, collidine and butanol-acetic acid mixture in which the unknown substance was compared directly with authentic glucosamine, and these confirmed the identity.

The only naturally occurring ninhydrin-reacting substance known to have R_F values close to glucosamine in these three solvents is chondrosamine, but separation may be obtained between these two amino-sugars on filter-paper chromatograms by pro-

longed irrigation with collidine (cf. Aminoff & Morgan, 1948). A few trials were required in order to determine satisfactory conditions for the separation of the two amino-sugars, and the following procedure was finally adopted. A strip of Whatman no. 1 filter paper was cut to a point at one end and spots of glucosamine hydrochloride, chondrosamine hydrochloride, the unknown mixture and a mixture of the authentic amino-sugars were applied to the starting line. When the spots were dry, a drop of concentrated ammonia solution was put on each of the spots and allowed to dry off. The chromatogram was then irrigated for 42 hr. with collidine, allowing the solvent to drip off the pointed end of the paper. On drying and spraying with ammoniacal silver nitrate or the Elson & Morgan (1933) reagents a clear separation was obtained between the two amino-sugars, and chondrosamine was seen to be absent from the unknown mixture.

Isolation of glucosamine and histidine from band VI of the primary separation

The filtration tube was 13 mm. in diameter and contained 10 g. (dry wt.) of 'Zeo-Karb 215' (80–100 mesh/in.). The column was prepared by alternate treatment with 0·15N-ammonia and 2N-hydrochloric acid in order to compact the bed (height occupied by the resin, 18·0 cm.). The mixed solution obtained from band VI of the primary separation (140 ml.) was applied to the column at a rate of 1·5 ml./min. and the column was then washed with 10–20 ml. water. Since the primary fractionation was carried out by displacement with 0·15N-ammonia the concentration of bases in the mixed solution of band VI was about 0·09M (cf. Part 1 of this series; Partridge, 1948), and thus the experimental mixture was sufficient to saturate rather less than half the new column (Part 1, fig. 3).

A dilute solution of sodium chloride (0·05M, 200 ml.) was applied at a rate of 2 ml./min. and the effluent was collected in 20 ml. fractions. A further quantity of more concentrated sodium chloride solution (0·1M, 400 ml.) was then applied, after which the column was washed with water (5–10 ml.) and the reservoir changed for one containing 0·15N-ammonia. This solution (150 ml.) was applied at a rate of 1 ml./min. and the effluent collected in 10 ml. fractions.

Fig. 2 shows the filter-paper partition analysis of the effluent fractions. A good separation was obtained between glucosamine and leucine and between histidine and lysine, but the histidine fraction was contaminated by a little cystine. A series of very faint spots due to phenylalanine was noticed on the paper chromatogram. The bulk of this amino-acid was removed by the preliminary charcoal treatment of the protein hydrolysate and it had not been observed in previous chromatograms carried out on the experimental solution; however, its appearance in the histidine band was probably due to the concentration of small traces by the chromatographic procedure. Fractions 9–12 (glucosamine hydrochloride) and 43–46 (histidine) were set aside for concentration and crystallization.

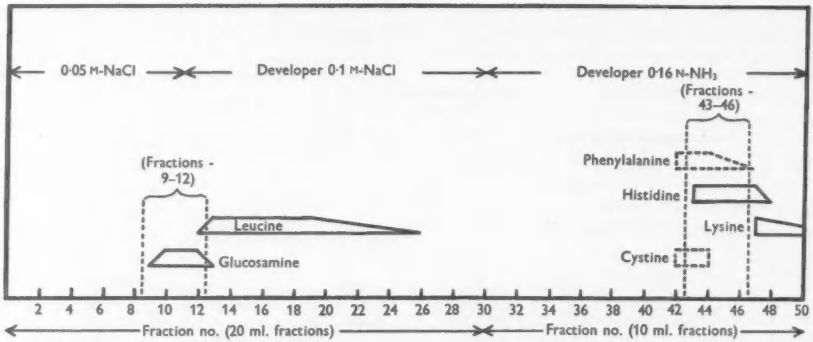

Fig. 2. Further fractionation of the histidine-glucosamine band obtained in the primary separation of the protein hydrolysate. The chromatogram shows the result of displacing the mixture from a column of 'Zeo-Karb 215' with an increasing concentration of NaCl followed by ammonia solution.

Crystallization of glucosamine hydrochloride. The mixed fractions 9–12 (80 ml.) were evaporated to small bulk under reduced pressure and the syrup diluted with about 4 vol. of methanol. The solution was then warmed, and warm acetone added until crystallization commenced. On cooling, a heavy crop of crystals separated (small prisms). Yield, 0·157 g.; ash, 0·7 %; N, 6·40 % (calc. for $C_6H_{14}O_5NCl$, N, 6·53%); $[\alpha]_D^{20^\circ} + 74$ (1 dm.; *c*, 1·5 in water); yield as percentage of protein dry wt., 0·47. A chromatographic analysis of the product showed it to be free from amino-acids.

Crystallization of histidine. The mixed fractions 43–46 (40 ml.) were concentrated to about 10 ml. under reduced pressure and the solution stored at +2° overnight. It was then filtered to remove a light precipitate which was probably cystine. A small amount of charcoal was added and the solution warmed. It was then refiltered and the evaporation continued until a few crystals appeared in the liquid. An equal volume of warm ethanol was then added and a dense feltwork of crystals appeared on cooling. Yield, 0·303 g.; ash, 0·3%; N, 26·5% (calc. for $C_6H_9O_2N_3$: N, 27·1%); $[\alpha]_D^{20°} - 32·6$ in water (*c*, 2·3; 1 dm.); yield as percentage of protein dry wt., 0·92. Chromatographic analysis showed the product to be free from other amino-acids.

DISCUSSION

The separation described above illustrates a point that has already been stressed in Part 3 of this series (Partridge, 1949), namely, that fractionation by displacement from an ion-exchange column has an advantage not possessed by the process of distillation from a fractionating column which it otherwise resembles: in the chromatographic technique it is often possible to alter the adsorption affinity of a solute by altering the environment in which the adsorption takes place; thus a primary separation may be carried out in order to divide the components into a series of small groups, and each group may then be separated further by a secondary fractionation carried out with an alteration in experimental conditions calculated to produce a differential alteration in adsorption affinity.

Two distinct methods of using ion-exchange resins in displacement chromatography have been described here, displacement with a free base and displacement with a salt; the substitution of one method by the other leads to a remarkable change in the order in which the components leave the column. The differential technique has been used in this case to separate a univalent base, glucosamine, from a complex ampholyte, histidine; but, although no further experiments on these lines have yet been attempted, it is clear that a similar technique could be applied to other separations in which the components are either different in charge structure or are well separated in a homologous series.

The isolation of glucosamine from a protein containing it in small amount has a special interest since many proteins are known to contain small quantities of hexosamines. Isolation by conventional procedures is a difficult matter, and although the hexosamine content of a protein may be estimated readily by colorimetric or other methods, none of these differentiates one hexosamine from another.

The yields of both glucosamine and histidine obtained in these experiments were low, probably not exceeding 40% in either case, but a large part of the loss occurred during the final process of crystallization which was carried out with 150–300 mg. of material. Higher yields should be obtainable by increasing the scale.

SUMMARY

1. Glucosamine has been identified as a constituent of the hydrolysis product of commercial egg albumin.

2. A primary fractionation of the hydrolysate carried out by displacing the components of the mixture from a column of 'Zeo-Karb 215' by means of dilute ammonia solution yielded a mixed band containing glucosamine and histidine together with other amino-acids in small amount.

3. The components of the mixed band were again displaced from a smaller column of 'Zeo-Karb 215' using dilute sodium chloride solution as the displacement developer. Glucosamine was displaced and was recovered in a pure condition as the hydrochloride. Histidine remained in the column.

4. The histidine was displaced from the column by means of ammonia solution and was recovered as the free base in a substantially pure condition.

5. The principle underlying the separation is discussed briefly and possible applications to other systems are pointed out.

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Enzyme Activities in the Blood of Infants and Adults

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Morphological studies of the blood of infants and adults have been fairly numerous, but functional or chemical comparisons have been rare. The circulating blood at birth has been shown to contain two kinds of haemoglobin, an infantile type which comprises four-fifths of the whole and which is relatively resistant to alkaline denaturation, and the adult type which makes up the remaining fifth (Haurowitz, 1930, 1935; Brinkmann & Jonxis, 1935). Immunological differences between the adult and infant haemoglobins were detected by Darrow, Nowakovsky & Austin (1940). Whitby & Hynes (1935) found that concentrations of sodium chloride, which caused no haemolysis of adult red cells, readily haemolysed a proportion of the cells of a sample of infant blood. A concentration of sodium chloride, however, sufficiently dilute to cause total haemolysis of normal adult cells, did not disrupt all the cells in a sample of infants' blood. Such samples therefore contained some cells which were more and others less fragile than normal adult cells. Mollison (1948) compared the survival time in the infant circulation of red cells taken from cord blood with that of cells taken from an adult vein. He found that cord red cells disappeared at nearly twice the rate of the adult cells during the 10 days following transfusion. Stevenson (1943) estimated the carbonic anhydrase activity of infant and adult red cells and found it to be low in the former, and still lower in red cells obtained from premature infants, and Anselmino & Hoffmann (1931) claimed that the catalase activity of red cells derived from full-term infants was higher than that of maternal red cells. If all the morphological, physiological and chemical evidence is taken together it would appear that the red cells of an infant are a more heterogeneous population than those of an adult.

The present work was undertaken to compare the enzyme activities of the serum and erythrocytes of the newborn and adult human. The true and pseudocholinesterase of the serum have been investigated, and in the erythrocytes true cholinesterase, glyoxalase, carbonic anhydrase, catalase and acid phosphatase.

METHODS

Preparation of serum and red cells. Blood was taken from the placental end of the divided umbilical cord or by puncturing the umbilical vessels with a large bore needle. Two samples were collected in sterile glass tubes or small bottles, one for the preparation of serum and the other, received into a few ml. of a solution containing 1 g. of sodium citrate dissolved in 100 ml. 0·9% (w/v) NaCl, for the separation of red cells. The specimens collected to provide serum were centrifuged as soon as the fibrin clot had separated. Haemolysed sera were not investigated. The citrated sample was centrifuged and the plasma removed. The cells were washed once with an equal vol. of 0·9% (w/v) NaCl, suspended in 0·9% NaCl and covered with a layer of liquid paraffin. In all manipulations care was taken to avoid bacterial contamination. Both the cells and the sera were stored in the dark at +4° until required. Keilin & Wang (1947) have shown that the activity of the enzymes under investigation was reduced by a very small amount when blood was similarly stored for many years, and no evidence of any loss of activity was found in the present work.

Cholinesterase was estimated in Warburg manometers at 37°. Two substrates were used: (*a*) 0·135 M-acetylcholine chloride, and (*b*) 0·18 M-acetyl-β-methyl choline. The latter is said to be hydrolysed only by the so-called true cholinesterase (Mendel, Mundle & Rudney, 1943). Since pseudocholinesterase in human blood is thought to be confined to the serum, and the true enzyme almost entirely to the red cells, it seemed permissible to use the unspecific acetylcholine chloride as substrate for the serum enzyme.

The quantities of the reacting substances used to estimate cholinesterase activity are set out in Table 1. In each case

Table 1. *Quantities of reactants used in Warburg flasks to estimate blood cholinesterase activity*

Enzyme	Side bulb containing substrate	Main compartment	Unit of activity
Serum pseudocholinesterase	0·135 M-Acetylcholine chloride, 0·5 ml.	Serum, 0·1 ml.; *Ringer-bicarbonate, 0·9 ml.	μl. CO_2/min./ml. serum
Serum true cholinesterase	0·18 M-Acetyl-β-methyl choline, 0·5 ml.	Serum, 0·5 ml.; 0·03 M-$NaHCO_3$, 0·5 ml.	μl. CO_2/min./ml. serum
Red-cell true cholinesterase	0·18 M-Acetyl-β-methyl choline, 0·5 ml.	Lysed red cells, 0·2 ml.; *Ringer-bicarbonate, 2·3 ml.	μl. CO_2/min./mg. Fe

* Bicarbonate solution of Krebs & Henseleit (1932).

the manometers were gassed with a mixture of 5% CO_2 and 95% of O_2 (v/v), and equilibrated until two successive readings were constant. With the quantities of reactants given, CO_2 evolution was a linear function of time over the period of the experiment. Readings were taken at 2·5 min. intervals for 15 min. except when the true cholinesterase was being estimated in the serum. Readings were then taken at 10 min. intervals for a period of 1 hr.

Column 4, Table 1, defines the unit of activity employed. In the red cells iron was estimated by the method of Lyons (1927), described by McCance, Widdowson & Shackleton (1936), and the activity of the enzymes was calculated/mg. of Fe.

Glyoxalase activities were measured in Warburg manometers at 37°. 0·5 ml. of a 0·0139 M-solution of methylglyoxal, which had been neutralized in bulk with a few drops of Na_2CO_3, was used as substrate. The main chamber of the Warburg flask contained 0·1 ml. of diluted lysed red-cell solution, 0·65 ml. of Ringer bicarbonate (Krebs & Henseleit, 1932) and 0·25 ml. of 0·36% glutathione. The gas space was filled with a mixture of 5% CO_2 and 95% O_2 (v/v). After equilibration six readings were taken at 2·5 min. intervals over which period CO_2 evolution was linear. The results were based on the iron content of the sample and the activity was expressed in μl. CO_2 evolved/min./mg. Fe.

Carbonic anhydrase activity was measured by the 'boat' method of Meldrum & Roughton (1933). Experiments were conducted at 16·0±0·2°. The activity was calculated from the formula given by the authors, and the result was expressed in their arbitrary units/mg. Fe in the sample of red cells under investigation.

Catalase. George (1947) showed that in the reaction between catalase and H_2O_2, O_2 evolution takes place in two phases. An initial rapid phase, of about 30 sec. duration, is followed by a slower phase. He claimed that the rate of O_2 formation in the rapid phase was proportional to the enzyme concentration. These observations formed the basis of the method employed to compare the activity of catalase in adult and infant red cells. The Meldrum & Roughton (1933) 'boat' apparatus was used. 1·5 ml. of 0·2 M-phosphate buffer, pH 6·5, and an equal vol. of diluted lysed red-cell solution were added to one side of the boat, and 1·5 ml. of 0·4 M-H_2O_2 to the other. After a period for equilibration with temperature and pressure, the 'boat' was shaken violently in the apparatus and the O_2 evolution followed to completion manometrically. Experiments were performed at 16·0±0·2°. Experiments in which water was substituted for the red-cell solution showed an insignificant O_2 evolution. The blank value was therefore neglected. O_2 evolution over the initial rapid phase of the reaction was linear for about 30 sec. All the results were calculated from the pressure difference after 20 sec. The results are expressed/mg. of Fe in the solution of red cells under investigation.

Acid phosphatase was estimated in red cells by the technique of King (1947). The unit of activity used is a modification of that defined by King, Wood & Delory (1945) and represents mg. of phenol liberated under the conditions of the experiment in 1 hr./mg. Fe in the sample of red cells under investigation.

Haemoglobin was estimated by the method of Gibson & Harrison (1945).

RESULTS

A summary of the results obtained for the seven enzymes examined is set out in Table 2. The mean activity of each enzyme, with the exception of acid phosphatase, was lower in infancy than in adult life. The differences were statistically significant. In the case of the red-cell acid phosphatase there was no significant difference between the mean values of the adult and infant group.

The values recorded for carbonic anhydrase confirmed the finding of Stevenson (1943), who showed the activity of infant blood to be lower than that of the adult, and although the unit used by him was not identical with that of the present work the difference was in the same ratio (roughly 1/3). We also confirmed his observation that the activity of the red cells in some newborn infants is almost immeasurably low. He also stated that the carbonic anhydrase activity of the erythrocytes of premature babies (1500 g. or less) was low when compared with the corresponding values in full-term infants. In the present series the birth weights varied between 2150 and 4470 g., but there was no evidence that a low

Table 2. *Comparison of enzyme activities in serum and red cells of adult and cord blood*

Enzyme and unit of activity	Age and no. of samples	Mean activity and S.E.	t	Degree of significance (P)
Serum pseudocholinesterase. Unit = μl. CO_2/ml./min.	Adult 24 Infant 24	76·7 ± 2·82 50·2 ± 2·94	6·43	<0·001
Serum true cholinesterase. Unit = μl. CO_2/ml./min.	Adult 24 Infant 24	1·053 ± 0·055 0·827 ± 0·043	3·22	<0·005
Red-cell true cholinesterase. Unit = μl. CO_2/mg. Fe/min.	Adult 11 Infant 24	48·31 ± 5·49 32·90 ± 1·94	3·3	<0·005
Red-cell carbonic anhydrase. Arbitrary units/mg. Fe × 10^2	Adult 13 Infant 26	36·65 ± 2·51 9·10 ± 2·41	14·8	<0·001
Red-cell catalase. Arbitrary units/mg. Fe	Adult 13 Infant 27	27·67 ± 2·39 19·23 ± 1·58	2·89	<0·01
Red-cell glyoxalase μl. CO_2/mg. Fe/min.	Adult 7 Infant 9	555·0 ±75·2 281·0 ±34·2	3·57	<0·005
Red-cell acid phosphatase. Arbitrary units/mg. Fe	Adult 11 Infant 9	4·61 ± 0·423 4·84 ± 0·517	0·375	Difference not significant

The difference is considered to be statistically significant when $P<0·05$.

birth weight was associated with low, or for that matter with high, carbonic anhydrase activity.

Estimation of catalase showed that infant red cells had a lower mean activity than adult red cells, and did not confirm the work of Anselmino & Hoffmann (1931), who compared the catalase activity of cord blood with that of maternal blood. However, these authors used a method which involved the estimation of residual hydrogen peroxide by permanganate titration, which cannot be assumed to give results comparable with those given by the present technique. There is, for example, no evidence that the activity measured by permanganate titration is equivalent to that measured over the initial part of George's (1947) biphasic reaction, or that the catalase activity of maternal blood at term is the same as that of the non-pregnant adult.

DISCUSSION

The average haemoglobin found in twenty-two samples of cord blood was 15·6 g./100 ml. and the average value for the adults was 15·9 g./100 ml. The cord blood, however, contained 54·3 % of cells by volume and the adult blood 49·5 %. The enzyme activities of the cells are expressed/mg. of iron, but since the percentage of iron in infant and adult haemoglobins is thought to be the same (Best & Taylor, 1945; Haurowitz, 1930, 1935) the activities of the enzymes can be expressed/g. of haemoglobin without altering their relationships. If, however, the enzyme activities had been expressed/unit volume of red cells the differences found would have been magnified because the infant cells contained less haemoglobin/unit volume. Thus the mean activity of the erythrocyte cholinesterase in adults was 52·25 units/ml. of cells, as against 48·31 units/mg. of iron, and in infants 31·8 units/ml. of cells against 32·9 units/mg. of iron.

It is difficult to decide what significance should be attached to the low enzyme activities of the red cell at birth. It is important to recognize that the erythrocyte, even at this time of life, is a highly specialized cell. Any chemical activity which it manifests, therefore, must be either a legacy of the haemopoietic mechanism which formed it, or an expression of the metabolism of a mature cell. If the erythrocyte enzymes investigated are involved in the haemopoietic processes, variations in their activity may be the cause of some of the differences in chemical composition which are known to exist between the adult and the infant red cell. Differences in activity might also affect the rate of haemopoiesis, and Rittenberg & Shemin's (1946) work suggests that the lower activity of the infant enzymes need not necessarily imply a slower rate of haemopoiesis. But until the differences in chemical composition and the factors controlling haemopoiesis in the adult and the infant are better understood such arguments must remain largely speculative. If, on the other hand, the erythrocyte enzymes are involved in the maintenance of the cell while it is in the circulation, it should ultimately be possible to correlate the differences of activity which have been demonstrated in adult and infant erythrocytes with the survival of these cells in the circulation. On this hypothesis, indeed, lower activities in the infant erythrocyte might have been inferred from Mollison's (1948) observations that transfused cord red cells disappeared more rapidly from the circulation than did adult cells. The integrity of the red cell must depend on the preservation of its membrane, and in this connexion the observation of Paleus (1947) is interesting, for he claimed that the erythrocyte cholinesterase was localized in the membrane.

The foregoing discussion should not be considered to embrace carbonic anhydrase, since its function in carbon dioxide transport and in the 'chloride shift' is well established, although the significance of its very low activity in the infant red cell is still obscure.

So little is known of the physiological function of the serum cholinesterases, that attempts to interpret the present findings seem neither profitable nor justifiable.

SUMMARY

1. The mean cholinesterase activity of sera obtained from cord blood was 50·2 units for the 'pseudo' enzyme and 0·827 unit for the 'true' enzyme. The mean values for normal adult sera were 76·7 units and 1·053 units respectively. The differences were statistically significant.

2. The activities of the erythrocyte enzymes, true cholinesterase, glyoxalase, carbonic anhydrase, and catalase obtained from cord blood were found to be 32·9, 281, $9·1 \times 10^2$ and 19·23 units/mg. iron. The corresponding values for normal adults were 48·3, 555, $36·6 \times 10^2$ and 27·7 units/mg. iron. All differences were statistically significant.

3. The erythrocyte acid phosphatase activity was measured in cord blood and was found to be 4·84 units/mg. iron, the adult control group showed a value of 4·61 units. The difference was not statistically significant.

Estimations of enzymic activity in some of the controls were done by Miss A. O. Hutchinson to whom our grateful thanks are due.

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The Fate of Certain Organic Acids and Amides in the Rabbit

9. LOWER ALIPHATIC AMIDES

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In an earlier investigation, reported in Part 3 of this series (Bray, James, Ryman & Thorpe, 1948), it was shown that rabbit-liver extracts were only slightly active in hydrolysing formamide, acetamide, propionamide and phenylacetamide. It had previously been shown (Bray, Neale & Thorpe, 1946) that phenylacetamide was apparently completely hydrolysed in the intact rabbit, since its excretion products were qualitatively and quantitatively virtually the same as those of phenylacetic acid. In the case of the aromatic amides for which information is available (Bray, Thorpe & Wood, 1949*a*) there is reasonable agreement between results *in vivo* and *in vitro*, so that it seemed desirable to investigate the metabolism of aliphatic amides in greater detail.

These compounds have, in general, attracted little attention, probably because of the difficulty in determining their fate. Schultzen & Nencki (1872) concluded that acetamide was not hydrolysed in the dog. This was supported by Steinhausen's (1914–20) finding that in the phlorrhizinized dog 72 % of a dose of the amide was excreted unchanged over a period of 6 days. In the rabbit, however, acetamide appeared to be largely hydrolysed, since Salkowski (1877–8) could detect only a small proportion of unchanged amide in the urine, and Rudzski (1876) claimed that a large proportion of a dose was excreted as acetic acid. Pommerrenig (1902) reported that small doses were completely catabolized. Indirect evidence that acetamide is slowly and only partially hydrolysed in the rat has been obtained recently by Anker & Raper (1948) in feeding experiments with acetamide labelled with ^{13}C. Halsey (1898) found that formamide gave rise to as much urinary formate in the dog as did formic acid itself, and was therefore presumably completely hydrolysed, while Gonnermann (1902) found that both formamide and acetamide were hydrolysed by sheep liver and formamide by sheep kidney. The other amides studied here do not appear to have been investigated previously, except by Fiske (1923), who carried out a study of their hydrolysis by fasting cats, using urinary amide nitrogen estimation as the criterion of splitting. Acetamide was excreted over about 4 days, a total of 70 % of the dose appearing unmodified. With the straight-chain amides up to valeramide, the lag in excretion diminished and the extent of hydrolysis increased progressively with increasing molecular weight, *n*-valeramide being almost completely hydrolysed (95–96 %) within 24 hr. The extent of hydrolysis of propionamide was 55–57 % and of *n*-butyramide 74–80 %. The order of stability *in vivo* was the reverse of that towards alkali, with which reagent the degree of hydrolysis

decreases as the molecular weight increases. In our earlier study (Bray *et al.* 1948) we found that shorter chain aliphatic amides in general were much more readily hydrolysed by alkali than by liver extracts.

This paper reports the results of an investigation upon the amides of straight-chain saturated fatty acids from formic to valeric, in which the excretion of unchanged amides was estimated in terms of the increased ether-soluble acid formed as a result of hydrolysis; the hydrolytic effect of rabbit-liver extracts and slices was also observed.

EXPERIMENTAL

A. *In vivo* experiments

Methods and materials

Diet and dosage. The rabbits and diet were as previously described (Bray, Ryman & Thorpe, 1947). The amides were administered by stomach tube as solutions or suspensions in water and the acids as solutions of their sodium salts. At the dose levels used none of the compounds showed toxic properties.

Estimation of amides. Owing to the lack of suitable characteristic properties, the amides could only be estimated by a non-specific method. A procedure based on the liberation of NH_3 by alkaline hydrolysis was unsatisfactory and the method finally adopted involved estimation of the increase in the ether-soluble acid after hydrolysis.

Urine (50 ml.) was hydrolysed by addition of 10 N-NaOH (5 ml.) and heating for 2 hr. in a boiling water bath; the pH was adjusted to 8, using thymol blue as indicator, the urine cooled and the volume adjusted to 100 ml. The ether-soluble acid content was then estimated as below. By this method 95–102% of added amides were recovered from normal rabbit urine.

Estimation of ether-soluble acid. Ether-soluble acid was extracted as previously described (Bray *et al.* 1947). Since, however, all the aliphatic acids in this study are volatile, the ether in the 'boiler' was not evaporated as usual but titrated directly after the addition of water (20 ml.), thymol blue being the indicator. 'Fixing' the extracted acid by addition of excess standard NaOH before evaporation was undesirable in estimations on unhydrolysed urines, since it has been shown that an error may be introduced owing to hydrolysis of unchanged amide during evaporation (e.g. phenylacetamide, cf. Bray *et al.* 1946).

Table 1. *Excretion of aliphatic acids and amides by the rabbit**

Compound	Dose (g.)	No. of exps.	Percentage of dose estimated as ether-soluble acid in urine (Ranges in parentheses)		Estimated percentage hydrolysis in rabbit†
			Unhydrolysed	Hydrolysed	
Sodium formate	2–3	5	4 (0–7)	7 (4–12)	—
Formamide	2	3	5 (0–6)	43 (41–45)	60 (55–62)
	3	3	5 (2–8)	48 (47–50)	56 (54–58)
	4	2	2 (1–2)	33 (29–36)	69 (66–72)
		8	**Av. 3**	**43**	**61**
Sodium acetate	2–5	7	1 (0–4.	1 (0–7)	—
Acetamide	1·5	1	0	71	29
	2	4	1 (0–4)	65 (44–78)	37 (22–56)
	2·5	3	2 (0–5)	75 (58–87)	27 (13–47)
	3	4	0	80 (62–94)	20 (6–38)
	5	4	1 (0–3)	72 (62–88)	29 (12–41)
		16	**Av. 1**	**73**	**28**
Sodium propionate	2–3	4	0	0	—
Propionamide	2	4	3 (0–6)	47 (36–59)	56 (50–64)
	3	2	0	52 (48–55)	49 (45–52)
	4	2	0	48 (48)	52 (52)
		8	**Av. 1**	**48**	**53**
Sodium *n*-butyrate	2–3	4	0	0	—
n-Butyramide	2	3	0	8 (0–16)	92 (84–100)
	3	4	0	17 (12–24)	83 (76–88)
		7	**Av. 0**	**13**	**87**
Sodium *n*-valerate	2	4	0	0	—
n-Valeramide	1·5	2	0	6 (0–12)	94 (88–100)
	2	2	0	0	100
	3	2	2 (0–3)	5 (0–10)	97 (93–100)
		6	**Av. 1**	**4**	**97**

* The values given for the excretion of amides represent the total excretion, in some cases over periods up to 72 hr. after dosage (cf. Table 2).

† Calculated from individual values.

RESULTS

Ether-soluble acid of unhydrolysed and hydrolysed normal rabbit urine. The average ether-soluble acid excretion by individual rabbits ranged from 661 to 850 mg./24 hr., expressed as hippuric acid. The ether-soluble acid content of hydrolysed urine is conveniently expressed as a percentage of the value for unhydrolysed; it ranged from 148 to 200%. The average for 107 experiments was 171%; the average for individual rabbits ranged from 157 to 181%. The day-to-day variations in output for each rabbit was usually within ±10% of the mean values for both unhydrolysed and hydrolysed urines.

Excretion of aliphatic acids and amides by the rabbit. Table 1 summarizes the results obtained, taking the difference between the amount of amide administered and the total amount excreted unchanged as a measure of the extent of hydrolysis. The results with acetamide, propionamide, butyramide and *n*-valeramide are in general agreement with those of Fiske (1923), the degree of hydrolysis increasing with increasing molecular weight. Formamide is an exception, apparently being hydrolysed to a greater extent than acetamide. The possibility of some other type of catabolic process for formamide has not, however, been excluded. Proof of the presence of two of the amides unchanged in the corresponding urine has been obtained by isolation from chloroform extract (acetamide) or the ether extract (propionamide).

No evidence of the excretion of the acids was observed after the first 24 hr., but there was a delayed excretion of some of the amides beyond 48 hr. after administration of the dose. Table 2 shows the percentages of the doses of the amides excreted unchanged in 24 hr. periods after the dose. Here also we are in general agreement with Fiske (1923). The lag period decreases with increasing molecular weight, and hence with increasing degree of hydrolysis.

Table 2. *Rate of excretion of aliphatic amides by the rabbit*

Amide	Dose (g.)	Average percentage of amide excreted unchanged in 24 hr. periods after dose: 1st	2nd	3rd	Total
Formamide	2·0–4·0	27	8	4	39
Acetamide	1·5–5·0	62	6	4	72
Propionamide	2·0–4·0	37	10	0	47
n-Butyramide	2·0–3·0	13	0	0	13
n-Valeramide	1·5–3·0	3	0	0	3

B. *In vitro* experiments

Methods

Buffer solution. All the experiments were carried out at 37° at pH 7·4 in a phosphate buffer consisting of 0·2M-Na_2HPO_4 (8 ml.) and 0·2M-NaH_2PO_4 (2 ml.).

Preparation of digests. (*a*) Slices were cut from the liver immediately after removal from the animal and placed in the buffer. The required amount (8 g.) was made up to 10 ml. with buffer and added to the substrate in oxygenated buffer to make a tissue concentration equivalent to that of the extracts. The digests were incubated in large flasks filled with O_2.

(*b*) Extracts were prepared as previously described (Bray *et al.* 1948). Extract (10 ml.) was added to the substrate (0·01204M) in buffer (50 ml.). In experiments lasting more than 6 hr. it has been our practice to add $CHCl_3$ as a preservative. It will be seen in Table 3 that this retards the rate of hydrolysis, which was greater in 5 hr. without than in 20 hr. with $CHCl_3$. In general, the constancy of controls could not be relied upon for more than 6 hr. in the absence of preservative.

Estimation of amide hydrolysis. The NH_3 formed was estimated by the method previously used (Bray, James, Raffan, Ryman & Thorpe, 1949*b*). In each experiment a control consisting of tissue or extract and buffer without substrate was included. A digest containing *p*-nitrobenzamide was also always included as a check upon the amidase activity of the liver.

Stability of substrates. All the amides examined were stable in the buffer and no formation of NH_3 was observed when they were incubated with boiled liver extracts.

Table 3. *Hydrolysis of aliphatic amides by rabbit-liver extracts*

Percentage hydrolysis. (Ranges in parentheses. Superior figures against ranges indicate the number of experiments)

	With $CHCl_3$: 3 hr.	5 hr.	20 hr.	Without $CHCl_3$: 3 hr.	5 hr.
Formamide	4 (3–5)[7]	4 (3–5)[4]	4 (0–9)[6]	1 (0–3)[4]	4 (2–5)[4]
Acetamide	3 (0–4)[3]	—	3 (0–7)[3]	—	2 (1–3)[2]
Propionamide	1 (0–4)[5]	2 (0–3)[2]	5 (3–8)[4]	1 (0–2)[2]	3 (0–5)[2]
n-Butyramide	4 (1–6)[4]	6 (6)[1]	9 (3–14)[4]	8 (4–12)[3]	13 (6–18)[3]
n-Valeramide	11 (4–19)[7]	13 (4–27)[3]	39 (29–45)[4]	48 (34–83)[5]	55 (41–85)[5]
Phenylacetamide	2 (0–5)[9]	6 (3–8)[2]	7 (0–19)[8]	15 (8–20)[4]	22 (12–32)[4]
p-Nitrobenzamide	39* (17–49)[7]	46* (17–58)[7]	61* (29–81)[7]	71 (56–92)[11]	79 (67–92)[11]

* These values should not be compared with those given in a previous paper (Bray *et al.* 1948) which were obtained with 0·00687M-*p*-nitrobenzamide.

Hydrolysis of aliphatic amides by rabbit-liver extracts. The degree of hydrolysis is given in Table 3, which also shows the retardation due to use of chloroform as preservative.

Hydrolysis of aliphatic amides by rabbit-liver slices. The degree of hydrolysis is shown in Table 4.

Table 4. *Hydrolysis of amides by rabbit-liver slices*

Amide	No. of exps.	Percentage hydrolysis (ranges in parentheses) 2 hr.	5 hr.	6 hr.
Formamide	3	8 (4–13)	10 (6–17)	10 (6–17)
Acetamide	5	0 (0–2)	1 (0–3)	1 (0–3)
Propionamide	4	3 (0–6)	5 (3–7)	5 (3–7)
n-Butyramide	5	18 (15–22)	35 (31–39)	38 (35–43)
n-Valeramide	4	37 (30–43)	65 (49–75)	70 (52–81)
Phenylacetamide	5	12 (9–19)	28 (19–36)	29 (22–40)*
p-Nitrobenzamide	7	37 (30–50)	66 (53–76)	70 (56–82)

* Four experiments only.

Comparison of the hydrolysis of amides by rabbit-liver extract and slices. The percentages of *p*-nitrobenzamide, *n*-butyramide, *n*-valeramide and phenylacetamide hydrolysed by extract and by slices prepared from the same liver are given in Table 5. It will be seen that there are appreciable differences between the degrees of hydrolysis brought about by extracts and by slices. It is difficult to assess the significance of these differences for *p*-nitrobenzamide and *n*-valeramide, but for *n*-butyramide and phenylacetamide they are considerable. It may also be noted that with *p*-nitrobenzamide there are considerable differences in the initial rate of hydrolysis.

DISCUSSION

Table 6 summarizes the results of both *in vivo* and *in vitro* studies. The results of the former for acetamide, propionamide, *n*-butyramide and *n*-valeramide are in good agreement with those of Fiske (1923) for fasting cats, the degree of hydrolysis increasing with chain length and the lag in excretion decreasing. Formamide is an exception, being hydrolysed to a much greater extent than acetamide, although the rate of excretion is comparable. The lag in excretion (Table 2) is probably not related to delayed absorption of the amides, since Höber & Höber (1937) found that in rat intestine there was little difference between the absorption rates of

Table 6. *Hydrolysis of amides in the rabbit and by rabbit-liver slices and extract*

Amide	Percentage hydrolysis by Intact animal	Extract (5 hr.)	Slices (5 hr.)
Formamide	61*	4	10
Acetamide	28*	2	1
Propionamide	53*	3	5
n-Butyramide	87*	13	35
n-Valeramide	97*	55	65
Phenylacetamide	100†	22	28
p-Nitrobenzamide	100‡	79	66

* Based on recovery of unchanged amide.
† Only 71% of dose accounted for, but metabolism almost identical with that of phenylacetic acid (Bray *et al.* 1946).
‡ Bray *et al.* (1949*a*).

Table 5. *Comparison of hydrolysis of amides by rabbit-liver extracts and slices*

Exp.	Time (hr.)	Percentage of hydrolysis of *p*-Nitrobenzamide Extracts	Slices	*n*-Butyramide Extracts	Slices	*n*-Valeramide Extracts	Slices	Phenylacetamide Extracts	Slices
1	0·5	19	13	—	—	15	10	1	2
	2	48	39	—	—	36	33	6	9
	4	65	62	—	—	48	56	10	16
	6	71	71	—	—	54	66	12	22
2	0·5	23	7	—	—	7	7	—	—
	2	47	30	—	—	22	30	—	—
	4	63	47	—	—	38	44	—	—
	6	73	56	—	—	43	52	—	—
3	0·5	32	17	2	8	—	—	3	6
	2	62	50	7	22	—	—	11	19
	4	72	70	12	32	—	—	17	31
	6	88	82	16	38	—	—	20	40
4	0·5	37	—	2	6	—	—	—	—
	2	64	—	9	20	—	—	—	—
	4	77	—	15	34	—	—	—	—
	6	81	—	19	43	—	—	—	—

acetamide and *n*-valeramide. The explanation more probably lies in the rate of reabsorption of these compounds in the kidney. The earlier finding (Bray *et al.* 1948) that in the case of phenylacetamide there is less correlation between the degree of hydrolysis *in vivo* and by extracts *in vitro* than with aromatic amides is shown by the present investigation to apply to other aliphatic amides. The amides studied were, with the exception of *n*-valeramide, hydrolysed to a much greater extent *in vivo* than would be expected from their *in vitro* hydrolysis by extracts. It is, however, possible to offer an explanation for this discrepancy, taking into account the observed lag in excretion of formamide, acetamide and propionamide. In these three amides it may be suggested that the extra reaction time allowed by the delay in excretion compensates for the feeble hydrolytic power of the enzyme responsible. This factor does not operate with *n*-butyramide, but there is instead evidence of more efficient enzyme action provided by the experiments with slices. In these experiments (Tables 4 and 5) the conditions used presumably approximate somewhat more closely to those *in vivo*, and the results show that the hydrolytic activity is much greater than in extracts. With phenylacetamide the percentage hydrolysis observed *in vitro* is 22–29 % compared with an *in vivo* hydrolysis of 100 % (Bray *et al.* 1946). In the earlier investigation, however, it was shown that there was a delayed excretion of the metabolites of phenylacetamide in the rabbit. Thus in the case of all five amides there are secondary factors which may be interpreted as accounting to some degree for the discrepancy between the observed *in vivo* and *in vitro* results. It should, however, be remembered that the *in vivo* results for formamide, acetamide, propionamide, *n*-butyramide and *n*-valeramide were obtained by determination of the amount of amide excreted unchanged. In the case of *n*-butyramide and *n*-valeramide the *in vitro* experiments indicate that hydrolysis of these amides occurs, but there is no significant enzymic hydrolysis of the other three. This leaves open the possibility that these three amides may be metabolized by some process other than simple hydrolysis, or in some organ other than the liver. Whether the enzyme responsible for the hydrolysis of aliphatic amides is the same as that which hydrolyses aromatic amides is not yet certain. The evidence at present available indicates that the hydrolysis of the aliphatic amides is depressed to a somewhat greater extent by chloroform than is that of *p*-nitrobenzamide. The possibility that different enzyme systems may be involved is being explored.

SUMMARY

1. The metabolism of formamide, acetamide, propionamide, *n*-butyramide, *n*-valeramide and their parent acids has been studied in rabbits by estimating the extra ether-soluble acid in unhydrolysed and hydrolysed urines after administration of the compounds.

2. The percentages of the doses of the amides excreted unchanged were 39, 72, 47, 13 and 3, respectively.

3. There is a considerable lag in the excretion of formamide and acetamide and a smaller lag with propionamide.

4. The hydrolysis of the above amides and phenylacetamide by rabbit-liver extracts and slices has been compared. Formamide, acetamide and propionamide were only hydrolysed to a very small extent. The degrees of hydrolysis for *n*-butyramide. *n*-valeramide and phenylacetamide were: for extracts, 13, 55 and 22 %; and for slices, 35, 65 and 28 %, respectively.

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The Biochemistry of Locusts

2. CAROTENOID DISTRIBUTION IN SOLITARY AND GREGARIOUS PHASES OF THE AFRICAN MIGRATORY LOCUST (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F.) AND THE DESERT LOCUST (*SCHISTOCERCA GREGARIA* FORSK)

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The tremendous destruction which can be caused by locust plagues is widely appreciated, but, although locust research has attracted the interest of a large number of workers, the biochemical aspects of the problem have received little attention.

Uvarov (1921) was the first to appreciate the significance of the different forms of *Locusta migratoria*, and his theory of phases as applied to Acrididae which swarm is now universally accepted. In the *solitaria* phase locusts live in isolation; this implies that they are independent of their neighbours rather than that they are particularly scarce. In the *gregaria* phase hoppers (nymphs) occur in dense bands, keep close to each other, and march together. Gregarious adults form vast swarms which keep together even in flight. The two extreme phases are connected by a complex series of transitional forms which are descriptively named *transiens*. Factors which are known to influence the transition from solitary to gregarious forms and vice versa have recently been discussed by Uvarov (1947). The main factor controlling phase transformation is probably the population density; when solitary locusts are brought together they gradually change into the gregarious phase which is most strikingly evidenced by the insects becoming quite different in colour. The effect of crowding is in all probability to increase the activity of hoppers owing to frequent encounters with their neighbours. This increased activity appears to be the fundamental cause of the colour changes, for a solitary hopper maintained in isolation will also assume gregarious characteristics when stimulated to continuous exertion (Husain & Mathur, 1936).

After hatching, *L. migratoria* and *Schistocerca gregaria* (the two species under present consideration) normally go through five hopper stages, moulting at the end of each, and after about 22 days (at 32°) finally emerge as winged adults; under optimal conditions, from 2 to 4 weeks after emergence the adults become sexually mature (Uvarov, 1928).

Gregarious hoppers of *L. migratoria* are characterized in the early stages by their dark appearance which is broken by yellow or brown spots and stripes; in the later stages orange brown predominates, although there are still considerable regions of black pigmentation. At adult emergence there appears to be little black pigment and the colour is greyish brown, but on reaching maturity the males become bright yellow and the females dark brown. The overall colour pattern is very stable. The coloration of solitary *Locusta*, on the other hand, varies considerably; solitaries are generally lighter coloured than *gregaria* and tend to adopt the colour of their environment (Faure, 1932). They contain little, if any, black pigment and do not undergo colour changes at maturity.

Solitary and gregarious *Schistocerca* exhibit very much the same characteristics as do the corresponding *Locusta*, although the pigment pattern is different. The coloration of gregarious hoppers is very stable and in the later stages the insects are lemon yellow with very pronounced black patterns. Immature adults are generally pinkish with small dark spots, especially on the elytra, and they turn to yellow when maturity is reached. The colour of solitary *Schistocerca* is much more variable than that of gregarious *Schistocera*; in general, hoppers are green, immature adults greenish white, and mature adults grey (Uvarov, 1928). Some appreciation of these colour variations in *Schistocerca* and *Locusta* can be obtained by examining the coloured plates produced by Faure (1932).

It will be obvious from the above very brief survey that investigations into the biochemistry of locust pigmentation are essential to a deeper understanding of the phase transformation. The pigments which are stated to occur in locusts are melanin (Chauvin, 1937, 1939*a*), acridioxanthin, a pigment of unknown constitution (Chauvin, 1938*a*, *b*, 1939*b*, 1940, 1941*a*, *b*, 1944), carotenoids (Lederer, 1935; Chauvin, 1941*b*; Goodwin & Srisukh, 1948, 1949), an unidentified green pigment variously described as a chlorophyll derivative (Chauvin, 1939*a*) and as a flavone (Chauvin, 1941*b*), flavins (Gourevitch, 1937; Drilhon & Busnel, 1939; Busnel & Drilhon, 1942; Drilhon, 1943), and, possibly, a pterin (Busnel & Drilhon, 1942).

In the present investigation attention has been confined to the two carotenoids, β-carotene and astaxanthin, which occur in both species (Goodwin & Srisukh, 1948, 1949), and the results of a study of

their quantitative distribution in *Locusta* and *Schistocerca* at various stages of development and in both the solitary and gregarious phases are now reported. A similar investigation on the distribution of acridioxanthin will be reported later.

MATERIAL AND METHODS

Two species of locusta have been studied: the African migratory locust (*Locusta migratoria migratorioides* R. & F.) and the desert locust (*Schistocerca gregaria* Forsk). The locusts were bred in captivity at the Anti-Locust Research Centre in London; they were maintained under reasonably constant conditions and fed entirely on grass and wheat bran. The only difference between the breeding of the solitary and the gregarious phases was that the solitaries were completely isolated (one in a 3 lb. jam pot of capacity about 1 l.), whilst the gregarious insects were reared in crowds (about 50/jam pot or at high densities in cages up to 150 l. in capacity). Specimens were sent to Liverpool by post and generally arrived in good condition; any dead insects were discarded. In order to determine pigment distribution the insects were dissected into three portions: head (comprising the head and pronotum), body and legs; the wings, which only contain relatively small amounts of carotenoids (Goodwin & Srisukh, 1949) were not examined systematically. The insects were killed with ether, although occasionally this step was omitted, and the heads and pronota snipped off with sharp scissors; any internal organs remaining attached to the head were removed with fine forceps, as was any residual food material in the buccal cavity. The legs and wings were removed from the bodies which were then slit ventrally and the internal organs removed. The various parts were weighed within 5–10 min. of dissection in order to reduce to a minimum errors likely to arise from drying of the tissues; this is especially necessary in the case of the body.

Extraction of pigments. Each portion was ground in a mortar with acid-washed silver sand and, if necessary, a small amount of anhydrous Na_2SO_4; the carotenoids were then extracted with cold acetone *in situ* by stirring carefully with a pestle. The acetone was decanted and filtered through a G4 sintered-glass filter. The residue was re-extracted with acetone, and the process repeated until no colour showed in the acetone; three extractions were generally sufficient.

Chromatographic separation of the carotenoids. The acetone was removed under nitrogen, and the residue immediately dissolved in a small volume (5–10 ml.) of light petroleum (b.p. 40–60°). This solution was run through a small column (10 × 2 cm.) of defatted bone meal (Mann, 1943; Goodwin & Morton, 1946; Glover, Goodwin & Morton, 1948), and the column washed with further small quantities of light petroleum until no more pigment was eluted; this fraction consisted of β-carotene only. The astaxanthin, sometimes contaminated by traces of carotenoid oxidation products, was then eluted with acetone. The volumes of the β-carotene and astaxanthin fractions were then suitably adjusted for spectrophotometric analysis.

Spectrophotometric analysis. Measurements of light absorption were made in a Beckman quartz photoelectric spectrophotometer. The measurements of both solutions (β-carotene and astaxanthin) were made at 450 mμ. The wavelength 450 mμ. was used for both solutions in order to reduce the time required to examine a considerable number of solutions. This wavelength is $\lambda_{max.}$ for β-carotene but not for astaxanthin; however, the $E^{1\%}_{1\,cm.}$ 450 mμ. for pure astaxanthin in acetone is known (*vide infra*).

Calculation of results. The $E^{1\%}_{1\,cm.}$ 450 mμ. values for the carotene and astaxanthin fractions for the various parts of the body were calculated using the expression $E^{1\%}_{1\,cm.} = E/cd$, where E is the observed extinction for a solution of c% in a cell d cm. thick. In order to obtain an accurate $E^{1\%}_{1\,cm.}$ value for the whole insect a weighted mean value of $E^{1\%}_{1\,cm.}$ was calculated, e.g.:

	Wt. of part (g.)	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.} \times$ wt.
Body	0·608	0·0233	0·01417
Head and pronotum	0·210	0·0193	0·00405
Legs	0·234	0·0178	0·00416
Total	1·052		0·02238

$$\text{Weighted mean } E^{1\%}_{1\,cm.} = \frac{\Sigma(E^{1\%}_{1\,cm.} \times \text{wt.})}{\Sigma \text{ wt.}} = \frac{0{\cdot}02238}{1{\cdot}052} = 0{\cdot}0213.$$

The amount of carotene or astaxanthin per insect can then be calculated using the expression:

$$\text{Amount } (\mu g.) = \frac{E^{1\%}_{1\,cm.} \text{ 450 m}\mu\text{. (obs.)} \times \text{wt.} \times 10^6}{E^{1,\%}_{1\,cm.} \text{ 450 m}\mu\text{. of pure pigment}}.$$

$E^{1\%}_{1\,cm.}$ 450 mμ. for pure β-carotene in light petroleum was taken as 2500 and for astaxanthin in acetone, 1968. This latter figure was obtained from the curve in acetone of spectroscopically pure astaxanthin (Goodwin & Srisukh, 1949) assuming that $E^{1\,cm.}_{1\%}$ ($\lambda_{max.}$) in acetone is the same as that in *cyclo*hexane, i.e. 2500 (Karrer & Würgler, 1943).

RESULTS

A very considerable mass of data has been accumulated and analysed, but in order to conserve space and simplify presentation only summarizing tables are presented. An important conclusion, which allowed the Tables to be considerably simplified, was reached when the complete data were considered. It was that there are no significant differences in the concentrations of the pigments in the body, head and pronotum, and legs, and that the relative amounts of pigment in these tissues (about 50, 25–30, and 20–25 %, respectively, of the total) merely reflect the relative weights of these parts. Because of this it has been considered unnecessary to record the results of analyses on the separate parts of the body, and only those referring to whole insects are presented and discussed.

In Tables 1–4 are collected the values for both concentrations and amounts of carotene and astaxanthin of whole insects at most stages of development of solitary and gregarious *Locusta* and *Schistocerca*.

The most important point which emerged from a study of these tables is that in both *Locusta* and

Table 1. *Carotenoid distribution in gregarious* Locusta

Stage	No. of insects examined	No. of batches examined	β-Carotene $E^{1\%}_{1\,cm.}$ 450 mμ. (petrol)	β-Carotene Amount/insect (μg.)	Astaxanthin $E^{1\%}_{1\,cm.}$ 450 mμ. (acetone)	Astaxanthin Amount/insect (μg.)
First	250	6	0·018	0·13	0·028	0·21
Second	105	4	0·023	0·38	0·052	0·97
Third	63	3	0·015	0·40	0·037	1·4
Fourth	34	3	0·028	2·1	0·021	1·8
Fifth	32	5	0·018	3·0	0·026	5·5
Immature (male)	19	7	0·029	8·4	0·0078	3·5
Immature (female)	27	7	0·027	10·7	0·0061	3·0
Mature (male)	35	9	0·029	15·4	0·0050	3·4
Mature (female)	30	9	0·036	35·8	0·0045	5·4
Immature (female) (blue)	4	4	0·0061	1·8	0·413	15·5
Mature (male) with protozoan infection	4	2	0·015	9·1	0·0077	5·8
Mature (female) with protozoan infection	7	6	0·0072	7·4	0·011	15·0

Table 2. *Carotenoid distribution in solitary* Locusta

Stage	No. of insects examined	No. of batches examined	β-Carotene $E^{1\%}_{1\,cm.}$ 450 mμ. (petrol)	β-Carotene Amount/insect (μg.)	Astaxanthin $E^{1\%}_{1\,cm.}$ 450 mμ. (acetone)	Astaxanthin Amount/insect (μg.)
First	46	2	—	—	0·029	0·22
Fourth	4	1	—	—	0·044	2·9
Fifth	7	2	—	—	0·030	5·0
Immature (male and female)	4	4	0·039	11·4	0·016	5·8
Mature (male)	3	3	0·035	19·1	0·010	7·1
Mature (female)	2	2	0·026	12·5	0·021	12·6

Table 3. *Carotenoid distribution in gregarious* Schistocerca

Stage	No. of insects examined	No. of batches examined	β-Carotene $E^{1\%}_{1\,cm.}$ 450 mμ. (petrol)	β-Carotene Amount/insect (μg.)	Astaxanthin $E^{1\%}_{1\,cm.}$ 450 mμ. (acetone)	Astaxanthin Amount/insect (μg.)
First	56	2	0·001	0·026	0·044	0·13
Second	36	3	0·031	0·63	0·34	0·72
Third	44	4	0·035	1·7	0·026	1·6
Fourth	22	3	0·016	1·7	0·028	4·1
Fifth	5	2	0·026	8·7	0·024	7·6
Immature (male)	12	5	0·038	22·9	0·016	12·5
Immature (female)	11	5	0·028	13·6	0·013	8·3
Mature (male)	6	3	0·069	44·0	0·0043	3·5
Mature (female)	2	1	0·061	55·8	0·014	16·5

Table 4. *Carotenoid distribution in solitary* Schistocerca

Stage	No. of insects examined	No. of batches examined	β-Carotene $E^{1\%}_{1\,cm.}$ 450 mμ. (petrol)	β-Carotene Amount/insect (μg.)	Astaxanthin $E^{1\%}_{1\,cm.}$ 450 mμ. (acetone)	Astaxanthin Amount/insect (μg.)
First	42	1	—	—	0·039	0·39
Fourth	3	1	0·042	5·5	0·053	5·6
Fifth	3	1	0·046	26·4	0·023	17·7
Immature (male and female)	5	3	0·045	25·3	0·014	10·1
Mature (male and female)	3	2	0·064	47·5	0·017	16·2

Schistocerca there is no difference in respect of carotenoid distribution between the solitary and gregarious phases; further, there is no difference between *Locusta* and *Schistocerca* in the concentrations of the pigments; but there is a tendency for adult *Schistocerca* to contain more of the pigments

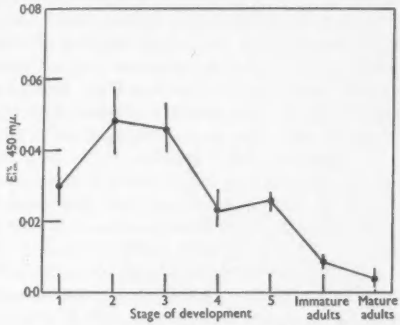

Fig. 1. The changes in the astaxanthin concentration ($E^{1\%}_{1\,cm.}$ 450 mμ.) of both phases of *Locusta* and *Schistocerca* during development. The vertical lines indicate the standard deviation of the results.

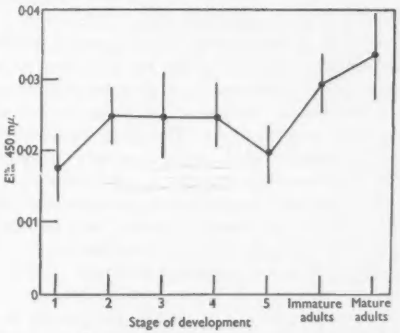

Fig. 2. The changes in the carotene concentration ($E^{1\%}_{1\,cm.}$ 450 mμ.) of both phases of *Locusta* and *Schistocerca* during development. The vertical lines indicate the standard deviation of the results.

than do adult *Locusta*; this is again merely a reflexion of the weight variations between the species. There is, however, a marked and important difference in the storage of the two pigments during development. As this difference obtains in both species, irrespective of phase, 'grand means' of the concentrations (measured as $E^{1\%}_{1\,cm.}$) and amounts of the two pigments have been calculated for each stage and plotted in Figs. 1–3.

Considering concentrations first, it will be seen (Fig. 1) that the astaxanthin concentration remains reasonably constant from birth to the third hopper stage, and thereafter diminishes until in mature adults it is not more than one-quarter to one-third of that of the early hopper values. With carotene the situation is different (Fig. 2); the carotene concentration at birth increases somewhat as soon as the insects begin to eat and then remains near a constant value until the insects become adult when the concentration increases until at maturity it is at least twice that at birth.

This difference between carotene and astaxanthin is reflected in the amount of the pigments present in the developing insects (Fig. 3). During the hopper stages the astaxanthin content increases gradually but rather slowly; the increase ceases at adult

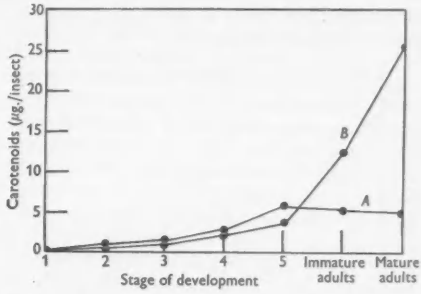

Fig. 3. The changes in the carotenoid content of locusts during post-embryonic development: *A*, astaxanthin content; *B*, β-carotene content.

emergence, and throughout adult life the levels remain constant at or just below the fifth-stage levels. The increase in β-carotene content in general parallels that of astaxanthin until the insects become adult when, in contrast to astaxanthin, it increases during adult life; this is especially marked after sexual maturity when considerable stores are found in the fatty tissues.

Carotenoids in the eggs. Newly laid eggs of both *Schistocerca* (Chauvin, 1941*b*; Goodwin & Srisukh, 1948, 1949) and *Locusta* (Goodwin & Srisukh, 1948, 1949) contain β-carotene only. In Fig. 4 are recorded the results of an investigation into the quantitative and qualitative changes in the carotenoid content of the developing *Locusta* egg. It will be seen that apparently β-carotene takes no part in the process of embryonic development until near the end of the period. It is only on about the 7th day that it begins to disappear and astaxanthin to appear; both these processes then continue until the insect is hatched. A similar experiment using *Schistocerca* eggs yielded very similar results, and is not recorded here.

It will be noted that the average carotene content of a *Locusta* egg is 0·38 μg. This means that an average egg pod of 50 eggs will contain about 20 μg. of β-carotene; the corresponding mean carotene level in *Schistocerca* eggs is about 1·0 μg./egg.

Carotenoids in exuviae. The appearance of carotenoids in the exuviae of any hopper stage has never been detected.

Carotenoids in dark blue Locusta. During the course of this investigation four atypical immature females were examined; they were very dark blue, had a metallic lustre, and were progeny of normal parents. However, it was noted that the subcutaneous fatty material was completely colourless, although one would have expected it to have been bright yellow owing to the presence of stored β-carotene. Chromatography of the carotenoid extract showed that the carotenoid present was almost completely astaxanthin; β-carotene was detected, but only in traces (see Table 1). The astaxanthin levels were much higher than normal.

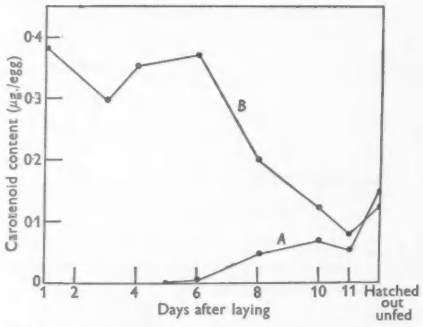

Fig. 4. The changes in the carotenoid content of fertilized *Locusta* eggs during embryonic development: *A*, astaxanthin content; *B*, β-carotene content.

Carotenoids in infected insects. It has been possible to examine locusts infected with a protozoan, probably *Plistophora*. The infected insects have a normal external appearance except that the outside of the abdomen becomes a characteristic pink. When they were opened it was found that the fatty tissues had lost their yellow colour and, except for the occurrence of pink spots, were almost white; it was therefore no surprise to find that, compared with healthy insects, the carotene contents of both males and females were very low; this was most marked in the case of heavily infected insects, especially females. On the other hand, the astaxanthin contents of both sexes were normal (Table 1). It was noted that the developing eggs of infected females still contained carotene, but it was not possible to ascertain the amount.

DISCUSSION

Chauvin (1941*b*) stated that the pink carotenoid recently identified as astaxanthin (Goodwin & Srisukh, 1948, 1949) is characteristic of immature locusts and that the yellow carotenoid (β-carotene) is characteristic of adults. This generalization can now be seen to have only a limited validity, but it did suggest the existence of an important difference between the metabolism of β-carotene and astaxanthin in hoppers and adult locusts. This difference has now been quantitatively demonstrated. At birth about 70 % of the total carotenoids is astaxanthin; this value gradually decreases until the fifth hopper stage; however, when the locusts become adult the accumulation of β-carotene proceeds at an enhanced rate whilst the amount of astaxanthin present remains stationary. This results in astaxanthin being never more, and often much less, than 30 % of the total carotenoids in adult insects.

It is not known why astaxanthin storage ceases when locusts become adult. It may be due to the fact that the rate of synthesis is reduced or stopped altogether, or that synthesis continues at the same rate, but that adult locusts utilize (or destroy) the pigment to a greater extent than do hoppers. It is interesting to note that wings, which are fully formed only in adults, contain relatively more astaxanthin than carotene; however, as they contain relatively little pigment compared with the rest of the body (Goodwin & Srisukh, 1949) it is not possible to postulate that astaxanthin synthesis in adults is diverted to the wings rather than to the body.

Formation of astaxanthin. This pigment, which is not present in the food of the locust, must be produced in one of three ways: (*a*) *de novo*, (*b*) by oxidation of alimentary carotenes, or (*c*) by oxidation of alimentary xanthophylls. The first possibility is almost certainly ruled out, for a survey of carotenoid biochemistry reveals no authenticated instance of carotenoid synthesis in animals (Goodwin, 1949*a*). Up to the present it has not been possible to decide which (if not both) of the other alternatives operates, for, as no adequate artificial diet is yet available, it has not been possible to carry out feeding experiments. No plant xanthophylls are ever found in locusts outside the alimentary tract, so that these pigments might either be converted quantitatively into astaxanthin or be quantitatively excreted; the latter possibility was put to experimental test. The relative amounts of carotenoids and xanthophylls were determined in *Locusta* faeces collected at all stages of development, together with the grass upon which they were feeding. It was considered that if only xanthophylls were found in the faeces or if the faecal xanthophyll/carotene ratio was very much higher than that of grass then it would be strong presumptive evidence that xanthophylls were preferentially excreted. However, it was found that locust faeces always contained a considerable amount of undigested or partly digested grass and were thus always very rich in both xanthophylls and carotenes, which occurred in ratios not differing significantly from those of the ingested grass. It was

therefore impossible to reach any conclusions as to the ability of the locust to absorb xanthophylls.

There is one fact which favours the suggestion that carotene is the precursor of astaxanthin. Astaxanthin is formed during the incubation of the egg, and, as it is unlikely to be produced *de novo*, carotene appears to be the only possible precursor.

Carotenoids in the developing egg. It has been shown quite clearly that β-carotene is used up during the later stages of embryonic development and that astaxanthin is formed. As has just been suggested, it is likely but not certain that astaxanthin is formed by the oxidation of β-carotene; even if this were so the astaxanthin produced does not account for all the β-carotene which disappears. What function the β-carotene plays in the developing locust egg is not known. Although carotenoids are often preferentially stored in the eggs of lower animals, little is known of their function, and it appears that the present work is the first to demonstrate that β-carotene is actively metabolized during embryonic development of the egg. In contrast, it should be noted that Mann (1946) and Suomalainen (1939) have reported no loss of carotenoids during the development of hens' eggs.

Any function in locust eggs which may eventually be assigned to carotenoids is perhaps unlikely to be a general one, for a number of insects, amongst which is the orthopteran *Blatella germanica* (McCay, 1938; Bowers & McCay, 1940), can exist on a diet free from carotene or vitamin A.

As has been stated earlier, the amount of carotene in a *Locusta* egg pod, containing, on the average, 50 eggs, can amount to at least 20 μg. This is of the same order as the amount found in a female without eggs and in an adult male. It seems, then, that females absorb more carotene, perhaps by eating more food, to provide the required amount for their eggs, for these obviously do not obtain their carotene at the expense of the other body tissues. The results of a limited number of experiments indicate that *Locusta* and *Schistocerca* eggs contain respectively about 0·4 and 1·0 μg. of β-carotene/egg. This difference is due mainly to the difference in size, for the weight of a *Schistocerca* egg is about 0·01 g. whilst that of a *Locusta* egg is about 0·06 g. The concentration of carotene in *Locusta* and *Schistocerca* eggs, therefore, does not differ significantly, and is about 100 μg./g. of eggs (wet weight); this is of the same order but somewhat higher than the value for *Schistocerca* eggs reported by Brodskis (1944) and Brodskis & Rungs (1944) who obtained 95 μg./g. dry weight; assuming a value of 30 % for the dry weight of the eggs, this reduces to about 30 μg./g. wet weight. On the other hand, the carotene content of grasshopper (*Melanoplus bivattus* Say) eggs has been reported to be 246 μg./g. dry weight (Grayson, 1942; Grayson & Tauber, 1943).

Relationship between carotenoids and colour of locusts. It has already been stated that there are no significant differences between the carotenoid contents of gregarious and solitary locusts, but it will be interesting to consider the other important stage of colour differentiation; that accompanying the onset of sexual maturity. At this stage males become yellow and females dark brown. It has been established in the case of *Locusta*, at least, that acridioxanthin, the main pigment of locusts other than the carotenoids, is unchanged in amount, state of oxidation and position in the integument* (Goodwin, 1949*b*) in males and females. The experiments reported here (see Table 1) have shown that the concentration of carotene in both sexes is the same; the amounts in the females are somewhat greater owing to their greater size. It seems reasonable to assume that the yellow colour which the males assume when they reach maturity is due to a migration of part of their carotenoids to the outer layers of the cuticle, for acetone will dissolve out a considerable amount of carotene from a piece of yellow cuticle carefully freed from fat. Owing to technical difficulties histological confirmation of this has not yet been achieved, but it is interesting to note in this respect that in males infected with *Plistophora* (Table 1) the loss of carotene is less than in infected females. This could be explained by assuming that the cuticle carotene in the males was only slightly affected compared with that stored in the adipose tissues; a similar survival of 'functional' carotene was noted in the eggs of infected females. Chauvin (1941*b*) has previously claimed that the yellow coloration of mature *Schistocerca* is due to β-carotene.

Although the striking colour differences between solitary and gregarious phases cannot be due to differences in the carotenoid content of the insects as a whole, it is very likely that they are due to differences in carotenoid distribution especially near the cuticle.

Carotenoid distribution in blue locusts. In the atypical immature female *Locusta* the acridioxanthin content was normal (Goodwin, 1949*b*), but instead of containing a mixture of carotene and astaxanthin, the former preponderating, only traces of carotene were present, but astaxanthin was present in excess (Table 1); further, astaxanthin must have been present in the integument, for the fatty tissues of the body were colourless. It may be that the outstanding blue colour of these locusts was due to an integumentary astaxanthin-protein, such as occurs in the purple patches of lobster carapaces (Kuhn & Lederer, 1933). Such a (brown) astaxanthin complex has been shown to occur in the wings of normal locusts, but up to now has not been found in their

* The author is grateful to Dr R. J. Daniel, Department of Oceanography, University of Liverpool, for the histological examinations of the locust integument.

integuments (Goodwin & Srisukh, 1949). It is possible that these insects were mutants in which an enzyme was produced (or activated) which either converts β-carotene very efficiently into astaxanthin or oxidatively destroys it. Such genetic control of carotenoid metabolism in insects has been noted previously (Gerould, 1921; Uda, 1919; Przibram & Lederer, 1933).

The value of locusts as a nutritional source of carotene. The nutritional importance of locusts, especially in North Africa, cannot be overlooked (Grant, 1948); as the carotene content of a gutted, mature locust is at least 30 μg., the value for an intact animal will be higher owing to the presence of carotene in the alimentary tract. Our investigations on whole *Locusta* indicate that their carotene content varies from 35 μg. for mature males to 70 μg. for mature females with eggs. An average value for mature *Schistocerca* has been given (Brodskis & Rungs, 1944) as 256 μg./g. dry weight which, assuming 70% of water, reduces to 80 μg./g. wet weight and to about 120 μg./insect. It will be seen that locusts are as good a source of β-carotene as are most green vegetables. In fact, it is quite possible that humans would utilize 'locust carotene' rather better than 'plant carotene', for the locust pigment is associated with considerable amounts of fat and it is well known that the presence of fat generally improves carotene absorption (see e.g. Hume & Krebs, 1949). It is interesting to note that from the carotene viewpoint the higher price charged for well-developed females (Dubois, 1893) is justified.

SUMMARY

1. The distribution of β-carotene and astaxanthin has been studied in the solitary and gregarious phases of two locust species: *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk. The locusts were examined at most stages of development, and the relative distribution in body, head and pronotum, and legs was also studied.

2. In both species no significant differences in the carotenoid distribution in the three main body regions occur between solitary and gregarious insects; further, no differences were noted between the two species.

3. The relative amounts of astaxanthin and carotene alter during development. In the early hopper stages astaxanthin comprises about 70% of the total carotenoids, whilst in mature insects this drops to below 30%.

4. When the β-carotene content of the eggs is allowed for, no differences are found in the carotenoid concentration of male and female insects.

5. No carotenoids were detected in locust exuviae.

6. Newly laid *Locusta* and *Schistocerca* eggs contain β-carotene to the extent of about 100 μg./g. (wet weight). During the later stages of pre-embryonic development β-carotene disappears and astaxanthin is formed.

6. In four atypical *Locusta*, which were very dark blue, astaxanthin occurred in considerably increased amount and carotene only in traces.

7. *Locusta* infected with the protozoan *Plistophora* store very little carotene but normal amounts of astaxanthin.

8. The possible importance of locusts, which contain β-carotene (provitamin A) in amounts similar to those found in green vegetables, as a source of vitamin A in some native dietaries is indicated.

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The Enzymic Degradation of Adenosinetriphosphate

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The present investigation arose from the observation that samples of adenosinetriphosphate (ATP), freshly prepared from skeletal muscle, gave a satisfactory phosphate cleavage with myosin adenosinetriphosphatase, whilst samples stored as the barium salt at room temperatures were very deficient in this respect. By the use of a specific inorganic pyrophosphatase (Bailey & Webb, 1944) it was later shown (Bailey & Webb, unpublished) that such specimens contained as much as 12% of the labile phosphorus as inorganic pyrophosphate, indicating a breakdown of ATP to this latter substance and adenylic acid. It may be recalled that Lohmann (1928), before his characterization of ATP, considered the labile phosphorus of muscle to be inorganic pyrophosphate, and did in fact isolate the crystalline sodium salt. Later, he considered it to have arisen during the preparative procedure (Lohmann, 1931).

It is clear that ATP preparations decomposing in this way may show a satisfactory ratio of total phosphorus/labile phosphorus/total nitrogen, and other criteria of purity must be employed. For this purpose enzyme methods have been explored. The purity both of laboratory and commercial specimens of ATP has been assessed, and changes occurring during known conditions of storage have been followed.

EXPERIMENTAL

Enzyme preparations

Myosin adenosinetriphosphatase. Myosin was prepared in the conventional manner from rabbit muscle (see Bailey, 1942), and was five times precipitated from water. It was stored in 0·5 M-KCl at pH 7 and 0° in presence of a trace of toluene, and was efficient enzymically over a period of 3–4 weeks.

Myokinase. The method of preparation followed strictly that of Colowick & Kalckar (1943). The enzyme was stored at 0° as an ammonium sulphate filter cake, and dissolved in dilute veronal buffer (pH 7) before use. As a paste, the enzyme remains active indefinitely.

Yeast inorganic pyrophosphatase. The purified enzyme prepared by Bailey & Webb (1944) was found to be active after storing at 0° for 5 years. In the initial experiments, a new batch of crude enzyme was prepared from brewer's yeast. It was found that yeast autolysate, after dialysis, contained not only the very active pyrophosphatase, but also a weak, Mg-activated adenosinetriphosphatase. After submitting the autolysate to the first four stages of the fractionation procedure of Bailey & Webb (1944), this latter enzyme had disappeared, leaving a very active pyrophosphatase suitable for the present work. Although the original enzyme has been used throughout, the preparation of new enzyme does not require the laborious procedure first employed. Preparations must be stored as an ammonium sulphate-protein paste at 0°.

Potato apyrase. This enzyme, discovered by Berger, Colowick & Slein (unpublished; see Kalckar, 1944*a*), splits off two phosphate groups from ATP. In critical experiments, however, it was found that preparations made according to the directions of Kalckar (1944*a*) are feebly active with respect both to inorganic pyrophosphate and adenylic acid. Our findings are supported in a recent paper by Krishnan (1949), who has compared the substrate specificity of crude and purified enzyme. In high concentration, even the purified enzyme possesses nucleotidase activity, but not at concentrations which are still able to split off two phosphate groups from ATP. It would appear, therefore, that potato apyrase is mixed with other phosphatases, and the usefulness of this enzyme is somewhat limited.

Incubation conditions

General. After establishing the complete specificity of the enzyme preparations, three test systems were adopted: the splitting of ATP (*a*) by myosin adenosinetriphosphatase, (*b*) by myosin adenosinetriphosphatase in presence of myokinase, and (*c*) by yeast inorganic pyrophosphatase. Additional experiments with potato apyrase were sometimes carried out. In (*a*), only the terminal phosphate group is split, whilst in presence of myokinase the adenosinedi-

phosphate (ADP) formed dismutes to adenylic acid and ATP, so that in the limit, the whole of the ATP is converted to adenylic acid. With pure ATP, the phosphate split by (*a*) should be half that of (*b*), but if ADP is present initially as impurity, (*b*) > 2 (*a*). Supposing also that inosinetriphosphate (ITP) is present, then, in absence of ADP impurity, (*b*) < 2 (*a*), since ITP is degraded to inosinediphosphate (IDP) by adenosinetriphosphatase, but IDP is not attacked by myokinase (Kleinzeller, 1942). Indirect evidence for inorganic pyrophosphate impurity is also obtained if 2 (*a*) or (*b*) fall far short of the values calculated from the labile P.

Without exception, tests were carried out in presence of excess enzyme, and the hydrolysis followed with a constant amount of enzyme over varying periods of time. Temperatures did not exceed 25° to avoid inactivation (see below), but this possibility was always assumed, and additional enzyme was added to several tubes of a series towards the end of the incubation period.

Myosin and myokinase. A tube series must be used rather than a single incubation mixture, since the splitting of phosphate in presence of Ca activator gives rise to the progressive precipitation of Ca phosphate, and accurate sampling is impossible. Each tube contained 1 ml. 0·5 M-glycine buffer (pH 8·6 at 20°), 0·1 ml. 0·1 M-$CaCl_2$, 0·1 ml. myosin solution (0·5 mg. protein), Na-ATP solution (0·2 mg. labile P), diluted with water to 1·6 ml. Another series was set up simultaneously, containing, in addition, myokinase (equivalent to 5 mg. protein paste). The tubes were incubated at 25° and the reaction arrested by adding 0·5 ml. of 10% (w/v) trichloroacetic acid. After standing at least 1 hr. in ice water, the protein was filtered off through small plugs of cotton wool into 25 ml. standard flasks, and the tubes washed with two 1 ml. portions of 3% trichloroacetic acid. The free phosphate, here and below, was measured colorimetrically in a photoelectric colorimeter by the Fiske-Subbarow method.

Yeast pyrophosphatase and potato apyrase. The incubation conditions for the former have already been described (Bailey & Webb, 1944). For the potato enzyme, 0·05 ml. 0·1 M-$CaCl_2$ was added to 2 ml. 0·03 M-veronal (pH 7), followed by enzyme and ATP to a volume of 2·5 ml. With both enzymes deproteinization was unnecessary, and the reaction was arrested by addition of trichloroacetic acid to pH 1.

Adenosinetriphosphate preparations

Preparation. The general procedure followed that of Lohmann as described by Needham (1942). The dibarium salt, without drying, was acidified to pH 1 with HCl, separated from any insoluble residue, and precipitated as the monobarium salt with an equal volume of ethanol (cf. Kerr, 1941*a*). The dibarium salt was again prepared by dissolving the monobarium salt in water and neutralizing with NaOH in presence of a slight excess of Ba acetate. After centrifuging, it was converted to the monobarium salt, which was finally dried in ethanol, ether, and *in vacuo* overnight. All preparations were stored at 0°.

Decomposition. When Ba^{++} is removed as sulphate in the manner previously described (Bailey, 1942), there is a loss of 20% of the ATP which is adsorbed on the $BaSO_4$. This loss may be minimized in the following way. Approximately half the Ba^{++} is removed as sulphate at pH 0·5–1, and the resulting solution run through a column of prepared Amberlite IR–100 (see Polis & Meyerhof, 1947; Kielly & Meyerhof, 1948). A solution equivalent to 0·2–0·3 g. of Ba-ATP can be employed for a 20 cm. column containing 3 g. of resin. The effluent emerges at pH 4·5, and is further neutralized to pH 6·5. (The resin is first activated by soaking in 5 N-HCl overnight, washed with water, then with 4% Na_2CO_3 and finally with water; the column may be recovered thereafter by washing with 2 N-HCl.) The removal of Fe^{+++} in ATP preparations by resin is only partial, and in this respect an acid column is more efficient than that described.

RESULTS

The labile phosphorus of adenosinetriphosphate. Following the procedure of Lohmann (1931) most workers have determined the labile P by hydrolysing in N-HCl for 7 min. at 100°. Both Fiske (1934) and Kerr (1941*a*), on the other hand, recommend a

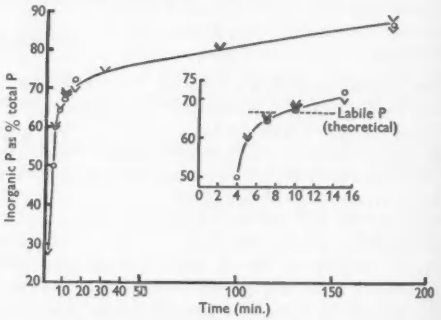

Fig. 1. Hydrolysis of ATP in N-HCl (boiling water bath; 1 ml. samples). Inset: part of curve enlarged. ▼, preparation 1 (3 experiments); ○ preparation 2 (1 experiment). Both samples prepared by author.

15 min. period. Whilst the shorter period of 7 min. seems just sufficient for inorganic pyrophosphate, it gives values low by about 3% in the case of ATP (1 ml. samples). If the degradation of ATP by acid occurs partly at the terminal P group and partly at the penultimate, the ADP and inorganic pyrophosphate thus formed will themselves follow a unimolecular course of hydrolysis which will be complete in some period > 7 min. The time at which labile P is just two-thirds of the total P will occur at a point where the P remaining in pyrophosphate linkage equals the phosphate contributed by the breakdown of adenylic acid (more strictly, of ribose-5-phosphate). This point, according to present findings (Fig. 1), is 9–10 min. after immersion of 1 ml. samples in boiling water.

Degradation of adenosinetriphosphate by myosin and myokinase. Fig. 2 illustrates the results of a typical experiment with a laboratory ATP preparation. A characteristic feature is the rapid initial splitting by adenosinetriphosphatase of 40% of the labile P (theory 50%), and thereafter a slow decomposition to the final value. The latter phase could

represent: (*a*) a low rate of hydrolysis at low substrate concentration, (*b*) inhibition by ADP, (*c*) inhibition by phosphate. The points lying near curve *A*, Fig. 2, show that ATP in low concentration, either alone or in presence of a large excess of ADP, is rapidly split, but that in presence of inorganic phosphate, the breakdown is relatively slow. The inhibition by free phosphate may be more apparent than real, since in the later stages of the reaction Ca phosphate is precipitated. The facility with which

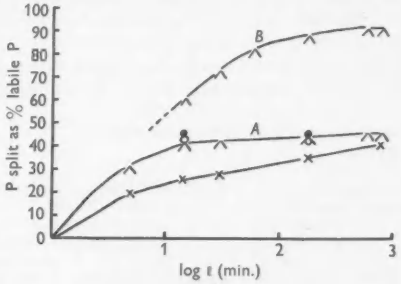

Fig. 2. Hydrolysis of a fresh specimen of laboratory ATP by enzymes. *A*, adenosinetriphosphatase; *B*, adenosinetriphosphatase-myokinase; each curve represents hydrolysis of ATP equiv. to 0·17 mg. labile P. ●, 0·05 mg. labile P of ATP without additions; ○, 0·05 mg. labile P of ATP + 0·23 mg. labile P of ADP; ×—×, 0·024 mg. labile P of ATP + 0·23 mg. inorganic phosphate; all in presence of adenosinetriphosphatase alone.

nucleotides form double salts makes it possible that some ATP is adsorbed by this precipitate and is less accessible to the enzyme. The addition of inorganic pyrophosphate in amounts sometimes found in ATP preparations (see below) has no significant effect upon the initial or final rates of splitting.

After 10 hr. at 25°, the splitting in the presence of myosin and myokinase (curve *B*, Fig. 2) is 92% of the labile P, and that with myosin alone (curve *A*), 47%. After this period, the enzymes are still capable of splitting added ATP, so that the cessation of hydrolysis must be due either to the attainment of an equilibrium, apparent or real, or to the presence of impurities. These points are more appropriately discussed below.

Inorganic pyrophosphate in adenosinetriphosphate preparations. Referring to his previous isolation of sodium pyrophosphate from muscle, Lohmann (1931) comments: 'The former investigations were in error, through the spontaneous decomposition of this compound (ATP) as the Ba salt in neutral or weakly alkaline solutions.' This warning as to the instability of ATP seems generally to have been ignored. When tested with yeast pyrophosphatase, old preparations of ATP which contained labile phosphate unavailable either to myosin or for the phosphorylation of glucose by yeast hexokinase, were found to contain inorganic pyrophosphate (Bailey & Webb, unpublished). The unique affinity of pyrophosphatase for its substrate gives a progress curve which is linear almost to the point where substrate is exhausted (Bailey & Webb, 1944). This effect is shown again in Fig. 3 where progress curves

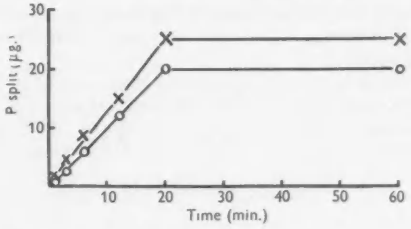

Fig. 3. Hydrolysis of inorganic pyrophosphate by yeast pyrophosphatase. ×—×, $Na_4P_2O_7$; ○—○, pyrophosphate in a stored commercial ATP preparation.

are given for pyrophosphate comparable in concentration to that found in a commercial ATP preparation which had been stored at room temperature for an unknown period. The absolute specificity of the enzyme, and the similar slopes, make it certain that the component degraded is inorganic pyrophosphate.

Table 1. *Enzymic assay of inorganic pyrophosphate produced during storage of adenosinetriphosphate samples*

ATP sample	Date of testing	Inorganic pyrophosphate (% of labile P)	Rate of breakdown (% of labile P/month)
Commercial (monocalcium salt)	1 Feb.	1	
	21 Sept.	12	1·5
Commercial (monobarium salt)	15 Jan.	2·5	
	4 May	5	0·7
Commercial (dibarium salt)	16 Jan.	2	
	21 Sept.	7·5	0·7
Commercial (monobarium salt)	26 Jan.	8	
	27 Nov.	19	1·1
Laboratory (monobarium salt)	26 Jan.	1	
	7 May	5	1·2
Laboratory (monobarium salt)	10 July	Nil	
	9 Sept.	1·5	0·8
Laboratory (solution of Na salt at −10°)	Dec. 1947	Nil	
	June 1948	Nil	Nil
Laboratory ADP (solution as above)	Dec. 1947	?	
	Oct. 1948	2·5	?
Mean for Ca and Ba salts			1·0

Both monobarium and dibarium salts, as also the Ca salt, break down progressively to pyrophosphate and adenylic acid on storage, even at 0°. Table 1 summarizes the pyrophosphate content of various

ATP preparations after storing at 0°. As a percentage of the labile P, the rate of production of inorganic pyrophosphate/month varies between the extremes of 0·7 and 1·5 %, mean 1·0 %. Whether the water content of the preparations influences breakdown has not been determined. In contrast, no pyrophosphate has been detected after storing solutions of Na-ATP at pH 6·5 in the frozen state even after 7 months, and only 2·6 % in a preparation of ADP similarly stored (this may have been present originally).

Analysis of some adenosinetriphosphate preparations. In Table 2 are collected chemical and enzymic analyses of laboratory and commercial ATP preparations. The ratio total P/labile P (each corrected for inorganic phosphate) is near the theoretical value of 1·5 in fresh laboratory specimens. Where the preparations have been stored, the free inorganic phosphate is high, and the ratio is only satisfactory if this latter is added both to labile and total P. This fact alone indicates some breakdown to ADP and phosphate, and the enzymic analyses support the inference; i.e. in these cases the splitting by myosin + myokinase is more than twice that with myosin alone, indicating the presence of free ADP. The breakdown of ATP to inorganic pyrophosphate on storage is thus accompanied by a less extensive decomposition to ADP and phosphate.

The labile P accounted for enzymically is never more than 93–95 %, even in laboratory preparations. Commercial preparations generally contain more free P and inorganic pyrophosphate, and show a lower rate of splitting with myosin-adenosinetriphosphatase. To check whether the enzyme assay gives a true measure of purity, the end products were isolated and again subjected to enzyme action. The assay by the muscle enzymes was also compared with the action of potato apyrase.

The action of potato apyrase on adenosinetriphosphate. Kalckar (1944*a*) records that the terminal group of ATP is split faster than that of ADP. The progress curve, given in Fig. 4, consists of two portions, the first representing mainly the splitting of the terminal phosphate of ATP, and the second that of ADP. A break occurs when most of the ATP is degraded to ADP and another when hydrolysis is complete. A somewhat similar curve was obtained by Albaum & Kletzkin (1948) with ATP from *Drosophila melanogaster*. The total splitting is 7 % above that obtained with myosin + myokinase, but of the 5 % inorganic pyrophosphate present, 2–3 % will have undergone hydrolysis by the pyrophosphatase present in the potato enzyme. Under the conditions of the experiment, the nucleotidase action can be neglected. (Initial hydrolysis rates of ATP, Na pyrophosphate and adenylic acid by apyrase are in the

Table 2. *Chemical and enzymic analysis of commercial and laboratory preparations of adenosinetriphosphate*

			Chemical analysis as percentage of total P*			Enzymic analysis as percentage of labile (10 min.) P		
Source	Type of salt	Total P (%)	Labile P	Free P	Ratio total P/ labile P	ATPase†	ATPase† + myokinase	Pyrophosphatase (yeast)
Author (fresh sample‡)	Monobarium	11·95	7·85	0·05	1·52	44·5	92	1
As above (stored 3 months)		—	—	—	—	43	86	5
Author (stored 6 weeks)	Monobarium	11·0	7·55	0·14	1·46	41·5	91·5	1·5
E.J.M.	Monobarium	—	—	—	—	46	92	1·5
Commercial (stored 1 yr.)	Monobarium	11·67	8·25	0·8	1·41	34·5	71	19
Commercial (fresh)	Dibarium	8·95	5·75	1·3	1·55 (1·45)	43	87	2
As above, stored 4 months		—	—	—	—	38	82	7·5
Author (stored 2 yr. at room temp.)	Dibarium	9·65	5·4	2·5	1·79 (1·53)	18	51·5	41
Advanced class (stored 5 yr. at room temp.)	Dibarium	7·76	4·72	1·55	1·64 (1·48)	Nil	2 (?)	95·5

* Total P and labile P are corrected for inorganic phosphate; figures in parentheses are derived from total P and labile P not corrected for inorganic phosphate (see text).
† ATPase = adenosinetriphosphatase.
‡ Atomic ratio P/N: calc. 3/5; found 3/4·96.

ratio 1, 0·01 and 0·003 respectively.) Thus, it would appear that the true ATP content is some 4 % higher than that indicated by use of the muscle enzymes. This figure is vindicated by experiments with ATP preparations containing no inorganic pyrophosphate, and by the isolation experiments below.

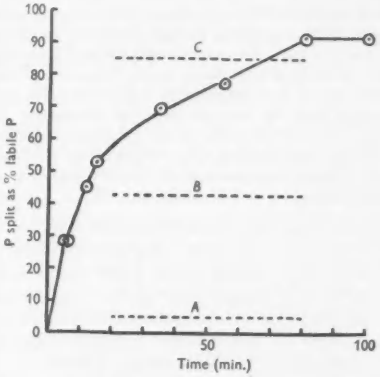

Fig. 4. Hydrolysis of ATP by potato apyrase. Dotted curves: *A*, level of splitting with yeast pyrophosphatase; *B*, with adenosinetriphosphatase; *C*, with adenosinetriphosphatase-myokinase.

Isolation of end products after the action of myosin or myosin plus myokinase

The ATP solution is incubated for about 7 hr. at 25° with myosin (or myosin + myokinase) under the standard conditions. The digest is adjusted to pH 5 with HCl, the precipitate of protein centrifuged off and the extent of hydrolysis determined on a sample of the supernatant liquid. An excess of Ba acetate (final concentration 0·1 %) is added and NaOH to a pH of 7, followed by ethanol to a final concentration of 50 % (v/v). After cooling to 0°, the Ba salts are centrifuged down, suspended in water, and HNO_3 added to pH 1. The nucleotides are now precipitated in the usual manner (cf. Needham, 1942) with the Lohmann mercury reagent, decomposed with H_2S and neutralized (after aeration) with Ba acetate and NaOH (final pH 7·5). After addition of an equal volume of ethanol, the Ba salts are separated, dried in ethanol and ether, and subjected to enzymic analysis.

Table 3 summarizes the results of three experiments, two with the end products after the action of myosin alone, and one after myosin + myokinase. The recovery of labile P in the case of the first two experiments is 60–80 % of the calculated amount, and most of the inorganic pyrophosphate present is recovered. The recovery of labile P from myosin-myokinase digests, on the other hand, is always low, and most of the inorganic pyrophosphate is now not precipitated with the Lohmann reagent. (This again illustrates how readily the nucleotides form double salts (cf. Kerr, 1941*b*).) The recovery of any undecomposed ATP is probably greater than the overall figures for the total recovery of labile P, so that the values derived in columns (*b*) of Table 3, corrected for isolation losses, are maximal. They indicate that the assay of ATP by the muscle enzymes is 1–2·5 % low. In the best laboratory preparations, therefore, some 4–5 % of the labile P has not been identified (see Table 2).

A possible impurity was at first thought to be guanylic acid, which according to Kerr & Seraidarian (1945) is present in the trichloroacetic extracts of various tissues, though the amount in skeletal muscle (dog) amounts only to 3 % of the adenine nucleotides. This probably arises by

Table 3. *Isolation and analysis of the end products after enzymic breakdown of adenosinetriphosphate*

(All values expressed as percentage of labile phosphorus.)

	Exp. 1		Exp. 2		Exp. 3	
ATP sample	Laboratory		Commercial		Commercial	
Method of degradation	ATPase*		ATPase*		ATPase*-myokinase	
Original analysis:						
Split by adenosinetriphosphatase	46		33		36·5	
Split by adenosinetriphosphatase-myokinase	92		69·5		80·5	
Split by pyrophosphatase (yeast)	1·5		14·5		12·0	
Analysis of large-scale digest:						
Labile P split	45		33		80	
Labile P recovered as percentage of calc. value	79		61		5†	
Analysis of recovered end products:‡	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Split by adenosinetriphosphatase	4	2·4	2·5	1·8	2·5	1·2
Split by adenosinetriphosphatase-myokinase	88	—	64	—	14·5	—
Split by pyrophosphatase	4	—	25	—	45§	—

* ATPase = adenosinetriphosphatase.
† Recovery here on the basis of labile P other than inorganic pyrophosphate.
‡ Values (*a*) as percentage of labile P present in the isolated end products; (*b*) as percentage of original labile P of digest after correction for isolation losses.
§ Of the original inorganic pyrophosphate 95 % was recovered from the water-soluble Hg salts.

degradation of tissue nucleic acid in trichloroacetic acid (cf. Kaplan & Neuberg, 1944). When ATP is hydrolysed for 10 min. in N-HCl, a substance is liberated which gives a blue colour with the Folin phenol reagent in alkali, though the original ATP contains no chromogenic material. If the latter were a chromogenic purine (guanine or xanthine) liberated from glycosidic linkage, the amount should remain constant after 15 min. when hydrolysis is virtually complete. It was found, however, that the amount steadily increased with continued hydrolysis, and that pure adenine itself was transformed in the same way. This reaction is worthy of further study.

Control experiments had shown that under the conditions of incubation of ATP in alkaline glycine buffer, no degradation to inorganic pyrophosphate or free phosphate occurred. The possibility that some of the missing labile P had arisen by the *enzymic* production of pyrophosphate was also checked. In these experiments, it was necessary to acidify the myosin-myokinase digests to pH 5 to inactivate adenosinetriphosphatase, to reneutralize to pH 7, add veronal buffer pH 7, and to swamp the Ca^{++} with a tenfold concentration of Mg^{++} to allow the action of pyrophosphatase, which is antagonized by Ca^{++} (Bailey & Webb, 1944). The difference in free phosphate before and after addition of pyrophosphatase was always within the experimental error. It was also shown that digests hydrolysed to the fullest extent, either by myosin or myosin-myokinase, and then subjected to 10 min. hydrolysis in acid, contained the amount of labile P added at the beginning of the experiment. None is lost, therefore, by transfer to a more stable form.

Adenosinediphosphate in muscle. Since ADP functions as phosphate acceptor in several reactions of the glycolytic cycle, and also as the substrate for myokinase, it seems unlikely that any appreciable concentration ever exists in mucle. Trichloroacetic extracts of fresh and rigor muscle were examined for the presence of ADP (*a*) by analysing the Ba salts precipitated at pH 7 and again after removal of free phosphate (*b*). No evidence of ADP in identifiable amounts was found in these crude fractions (Table 4). Method (*a*) suffers from the disadvantage that the large amounts of free phosphate present make enzymic analysis difficult, whilst in (*b*) some ADP may be lost in the ethanol-HCl procedure used for the removal of phosphate. The presence of a small amount of ADP can, however, be shown in the mother liquors obtained after precipitating ATP.

Table 4. *Enzymic analysis of the crude barium salts from trichloroacetic extracts of muscle*

Muscle (rabbit)	Mode of preparation	Phosphate split as percentage labile P: ATPase*	Phosphate split as percentage labile P: ATPase*-myokinase
Fresh	Ba salts pptd. at pH 6·5, washed twice	41	81
Fresh	As above, then pptd. in ethanol-HCl	45·5	91
Stood 5 hr. at 15°	Ba salts pptd. at pH 6·5, washed twice	35†	70†
Stood 5 hr. at 15°	As above, then pptd. in ethanol-HCl	44	88

* ATPase = adenosinetriphosphatase.
† Hydrolysis probably incomplete, owing to large amounts of inorganic phosphate present.

The once-precipitated Ba salt of ATP (11·9 g.) was dissolved in 30 ml. N-HCl, diluted with water and precipitated as the dibarium salt (total volume 1·2 l.). The precipitate was spun down, dissolved in HCl (total volume 500 ml., pH 2) and an equal volume of ethanol added. The combined mother liquors were neutralized to pH 7·5, an equal volume of ethanol added, and the precipitated Ba salt dried and decomposed. Enzymic analysis gave (as percentage of the labile P): split by myosin, 23%; by myosin-myokinase, 88%; by pyrophosphatase, 4%. Of the labile P, 42% thus existed as ADP, corresponding to 1·1% of the labile P of the original ATP.

Allowing that the starting material had been subjected to some purification, and that the final product might still contain some ADP, the true ADP content cannot exceed this figure greatly, since larger amounts (up to 5% of the total labile P) would be discovered by direct enzymic assay of the main ATP preparation. By contrast, Kalckar (1947) infers that considerable amounts of ADP are present in guinea-pig and hamster muscle.

DISCUSSION

The purity and stability of adenosinetriphosphate

The present results indicate that ATP preparations made by current modifications of the Lohmann method and precipitated 2–4 times are at least 95% pure. When freshly prepared they contain less than 1% inorganic pyrophosphate, which increases on storage at 0° at the rate of 1%/month. Both calcium and barium salts are subject to this change, and one sample of a dry sodium salt, stored at room temperature for a long period, had entirely decomposed. A less extensive breakdown to ADP and phosphate also occurs. If ATP samples have been prepared via the monocalcium or monobarium salt, the presence of free phosphate is itself indicative of extensive decomposition.

The complete enzymic assay of ATP preparations before use for other purposes may not always be possible. In this case, it is advisable to use ATP samples of known history and to check the extent of decomposition with yeast pyrophosphatase. This latter enzyme can be prepared in a form which is stable over many years, and the amounts required do not necessitate deproteinization.

It has not been possible to extend the investigation to the effect of water content on the decomposition of ATP salts, though negligible breakdown occurs when solutions of the sodium salt are stored at $-10°$. Recently, it has been found that ATP can be precipitated quantitatively as the benzidine salt

from aqueous ethanol at acid pH, and is quantitatively recovered by decomposing the complex with sulphuric acid, which forms the insoluble benzidine sulphate. The decomposition of barium-ATP, on the other hand, always involves some loss of nucleotide, whilst that of the acridine compound necessitates ether extraction. These advantages of benzidine as a precipitant for ATP will be enhanced if the complex is found to be more stable than the metallic salts of ATP.

The enzyme systems

Whilst the use of myosin adenosinetriphosphatase alone, and in conjunction with myokinase, is in theory ideal for the determination of mixtures of ATP and ADP, the practical application is more difficult than was at first realized (cf. Bailey, 1948*a*). The main disadvantage lies in the slow hydrolysis of ATP by adenosinetriphosphatase in the later stages of the reaction, when the substrate is almost exhausted and inorganic phosphate has accumulated. Previous workers (e.g. Rapoport & Nelson, 1945) who have used myosin for the assay of ATP in biological material do not seem to have been aware of this effect, which is probably due to adsorption of ATP on the precipitated calcium phosphate. The long incubations necessary for the assay of ATP by adenosinetriphosphatase (7–10 hr. at 25°) demand that the myosin be free from myokinase, and 4–5 precipitations are essential.

As an alternative to the use of myosin, potato apyrase is not entirely specific, and shows a feeble inorganic pyrophosphatase activity, and a still feebler action upon adenylic acid. Except for very precise analysis, these disadvantages are not serious. The usefulness of apyrase has been fully demonstrated by Kalckar (1944*b*), who has been able to differentiate the two labile phosphate groups of ATP in which ^{32}P had been incorporated. The terminal group was isolated by the yeast hexokinase reaction and both groups by the action of apyrase. The same differentiation or, if need be, the assay of mixtures of ADP and ATP, can be achieved by the use of hexokinase acting singly and in conjunction with myokinase (Kalckar, 1943). Unfortunately, the preparation of pure yeast hexokinase is a formidable task (cf. Bailey & Webb, 1948), and less pure preparations may be contaminated with adenosinetriphosphatase (Kielly & Meyerhof, 1948). Kalckar (1947) later made use of apyrase in the development of an optical micromethod for the determination of adenine compounds, basing the method upon the marked change in the ultraviolet absorption when adenine, free or combined, is deaminated to hypoxanthine. In this way adenylic acid can be estimated after the action of muscle deaminase, ADP by myokinase in conjunction with deaminase, and ATP by apyrase and deaminase.

Finally, some mention must be made of the claims of Banga (1947) and of Banga & Josepovits (1947*a*, *b*) with respect to the action of myosin on ATP. It is claimed that part of the ATP is degraded to ADP and part utilized in the formation of a dinucleoside pentaphosphate. The isolated dinucleotide shows some splitting in presence of myosin, but the production of inorganic phosphate is much greater in presence of myosin plus 'protein II'. The latter, as already pointed out (Bailey, 1948*b*), exhibits many of the properties of myokinase, and there is nothing in its manner of preparation which suggests its elimination. The obvious explanations for these results seem to have been meticulously avoided. First, in the action of myosin on ATP, only 33 % of the labile phosphorus is split, and the manner of isolation of the end products would give a product containing a mixture of ATP, ADP and inorganic pyrophosphate (if present). The renewed action of myosin itself on this mixture would affect only the ATP component, but acting in conjunction with protein II (myokinase), both ATP and ADP would be degraded to adenylic acid and inorganic phosphate.

Secondly, ammonia formation indicates the presence of adenylic acid deaminase in protein II. When the latter is added to the mixture, the ADP already present dismutes to ATP and adenylic acid by the action of myokinase, and the adenylic is at once deaminated. The deamination process itself speeds up the dismutation by removing one component of an equilibrium mixture, so that the initial formation of ammonia would be very rapid. This was actually observed. Later, ammonia production keeps pace with the splitting of phosphate, and this too can be explained, since the production of ADP, and hence of adenylic acid, is controlled by the hydrolysis of ATP by myosin, which becomes the limiting reaction. The overall reaction is

$$\text{ATP} \rightarrow 2\text{PO}_4''' + \text{NH}_3 + \text{inosinic acid.}$$

Expressed as a percentage of the total labile phosphorus and total amino nitrogen respectively, the liberation of free phosphorus and of ammonia must coincide. The results do not support the claim of Banga & Josepovits (1947*b*) that the action of myosin on ATP produces, in addition to the dinucleotide, an isomeric ADP in which a phosphate group is linked to the amino group of adenine. Not only are Banga's results unwarranted on the experimental evidence, but they can readily be explained in terms of known enzyme systems.

SUMMARY

1. The enzymic assay of adenosinetriphosphate and adenosinediphosphate, either singly or in mixtures, has been explored by use of myosin adenosinetriphosphatase acting alone and in conjunction with

myokinase. A disadvantage of the method is the slow hydrolysis of adenosinetriphosphate in the final stages of the reaction.

2. Metallic salts of adenosinetriphosphate are shown to be unstable and break down on storage to adenylic acid and inorganic pyrophosphate, and to a less extent to adenosinediphosphate and inorganic phosphate.

3. The best preparations of adenosinetriphosphate are at least 95% pure. The possibility that some impurity is present is not excluded.

4. The use of potato apyrase for the assay of adenosinetriphosphate is restricted by its contamination with a nucleotidase and an inorganic pyrophosphatase.

5. Both in fresh and in rigor muscle, the adenosinediphosphate present is less than 5% and probably greater than 1% of the labile nucleotide phosphorus.

6. The claims of Banga concerning the action of muscle enzymes on adenosinetriphosphate are criticized.

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Preparation of the Antibiotic Nisin

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The early pharmacological work with nisin, prepared from *Streptococcus lactis* (Lancefield group N) (Mattick & Hirsch, 1947) was remarkable for the success which attended the use of crude concentrates. Nevertheless, it was obvious that more potent material would be required for human therapy and for experiments with the less sensitive pathogens. A note (Berridge, 1947) added to the paper of Mattick & Hirsch showed that highly active preparations in the form of crystals or tactoids could conveniently be made. The details of a number of such preparations are now described.

METHODS

(1) *The unit of antibiotic activity.* A preparation of nisin powder was chosen as a standard, and its activity defined as 1·3 units/μg.; 1 unit will normally just inhibit the growth of the test organism, *Strep. agalactiae*, in 1 ml. of broth (test Ai), and is roughly equal to the unit of Mattick & Hirsch.

(2) *Measurement of antibiotic activity.* Three tests were given a prolonged trial during this work. A, Bacteriostatic tests: (i) dilutions of the antibiotic in powers of 2 in inoculated broth, with the recording of growth after 16 hr. and 40 hr. as +, + + or + + +; (ii) dilution of the antibiotic in powers of 1·3–1·5 in broth or skim milk heavily seeded with sensitive acid-forming organisms with the recording of pH after 10–16 hr. incubation; and B, an apparently bactericidal test in which the proportion of organisms surviving a few minutes' contact with the antibiotic diluted in powers of 4 was determined by the plate count. It is quite possible that different activities were measured by the different methods. The details of the methods are as follows:

Method A i. This did not differ significantly from that described by Mattick & Hirsch (1947).

Method A ii. The nisin samples were diluted on the basis of an assumed activity to give solutions with about 10 units/ml. Suitably spaced volumes (e.g. from 0·08 ml. increasing

by powers of 1·5 to 1·20 ml.) were measured from a micro-burette into a series of sterile test tubes. For each set of determinations carried out on the same day with the same preparation of test organism, a standard nisin solution freshly diluted to 10 units/ml. was dispensed similarly but over a smaller range and at closer intervals (e.g. from 0·1 ml. rising by increments of 0·03 ml. to 0·70 ml.). Pasteurized skim milk tinted with litmus was inoculated with 1% (v/v) of a 24 (±3) hr. culture of *Strep. cremoris* (strain 1P5) and 10 ml. mixed with the nisin solutions. The tubes were then incubated at 22°. The progressive reduction of the litmus by the growing micro-organisms, and the final clotting of the milk, enabled the state of each test to be observed at a glance. After about 10 hr. incubation pH determinations were made on each partly grown culture. From the graph of nisin concentration against pH, drawn from the standard, it was then possible to determine the activity of the unknown solutions. If incubation was too prolonged the graph from the standard test became too steep, there being then only one or two intermediate points between which to draw the useful part of the curve. The same technique has been followed using yeastrel dextrose broth in place of milk. The heavy inoculum, and the rapid growth of the test organism at a comparatively low temperature, made it possible to dispense with most of the usual precautions of asepsis, but samples were freed from gross contamination by being diluted in 0·05 N-HCl.

The uncertainties in constructing and using the standard graphs gave an error of less than ±10% in the final figures. The method was based on a suggestion of Hirsch, which he has since subjected to considerable study and elaborated into a 'lag-phase assay' (using *Strep. agalactiae*), the reproducibility of which has been statistically analysed (Hirsch, 1949).

Method B. A 0·001% suspension of a 24 (±3) hr. culture of *Strep. agalactiae*, giving a plate count of 3000–6000/ml., was allowed to stand for 6 min. in contact with serial dilutions of standard nisin diminishing from 50 units/ml. in powers of 2, and of unknown solutions diminishing from about 100 units/ml. in powers of 4; 0·1 ml. of each mixture was then plated with 10 ml. of melted cooled agar and counts made after 24–48 hr. incubation at 37°. Reductions of 30–70% in the count usually occurred between 6 and 50 units/ml.

A rough graph was made by joining with straight lines the points obtained when x was plotted against y, where $x = \log(100a/b)$, a = number of organisms surviving contact with nisin, b = number of living organisms before adding nisin, and y = nisin concentration in contact with the organisms in the broth suspensions. The activities in the unknown solution were then obtained by interpolation. Although the variations from day to day were great, and although there were factors of unknown magnitude such as the number of organisms killed in, for example, tube no. 2 before transferring 1 ml. to tube no. 3, it was anticipated that they would be overcome by the preparing of a standard graph for each set of determinations. However, this was not so, for after a few months' trial the method appeared only a little better than method Ai and was therefore abandoned in favour of Ai for ordinary and Aii for special purposes, although by this time it had been used for most of the experiments in purifying and crystallizing nisin. The plate counts of *Strep. agalactiae* have shown a reproducibility unusual with long-chained organisms. This and other aspects of the 'bactericidal action' of nisin have been examined in detail by Hirsch (1949).

At an early stage of the work the use of *Strep. cremoris* in a test like Ai led to the preparation of a concentrate active against this organism but without effect upon *Strep. agalactiae*. Therefore test Aii with *Strep. cremoris* has been used only when its different sensitivity was not likely to be misleading.

(3) *Determination of specific activity.* Samples, usually of 0·1 ml., of nisin solutions were dried at 98–100° in small platinum dishes, weighed on the microbalance, ashed, and reweighed as described elsewhere (Berridge, 1945). The specific activities were calculated in units/μg. of organic matter.

(4) *Determination of nitrogen.* The micro-Kjeldahl method of Tompkins & Kirk (1942) was used but with the digestion mixture of Campbell & Hanna (1937).

(5) *Apparatus for cooling at a controlled rate.* This was used for some of the later crystallizations.

A contact thermometer (electro-methods) in which the temperature setting could be controlled by rotating a magnet, was set up in a thermostatically controlled water bath. A train of Meccano gears and chain drives was then arranged between a synchronous electric motor and the controlling magnet so that the temperature of the water bath could be changed at any desired rate. The small fluctuation permitted by the contact thermometer appeared to be unimportant. With the help of a cooling coil connected to the main water supply, temperatures somewhat below room temperature could be reached.

(6) *Diffusion measurements.* Diffusion was allowed to take place through a sintered glass disk according to the technique described by Northrop & Anson (1929) (see also Gordon, 1945). Special precautions were taken to overcome vibration, and the temperature was regulated at 25±0·003°. The vessel was standardized with 0·1 N-KCl using the value for the integral diffusion coefficient $Di = 183{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. (Gordon, 1945). Measurements with nisin were made by comparing the activities of the inner and outer solutions according to method Aii.

(7) *Solubility determinations.* The nisin suspensions were allowed to reach equilibrium in bottle-shaped vessels of about 0·5 ml. capacity, each containing a newly made glass bead of smooth bright surface, which stirred the suspension while the bottle was rotated in a vertical plane. The suspensions were transferred to the bottles with a Pasteur pipette having a waxed tip. By filling exactly to the neck it was possible to avoid bubbles and at the same time to achieve adequate stirring. In order to avoid a partial vacuum in the bottles after sealing, the necks were first pulled out to very fine tips, allowed to cool and finally sealed with a minute flame. The sealed bottles were packed in cotton wool and immersed in crushed ice in a quart Thermos flask which was rotated in a vertical plane. After equilibrium had been reached, the suspensions were allowed to settle for several days and the supernatant solutions removed by pipette. This process was repeated to ensure freedom from contamination by traces of solid, and the nitrogen content of the solutions determined.

PREPARATIONS

Preparation of crude concentrates. The nisin-producing organism was a *Strep. lactis* (Lancefield group N) strain M354/07, identical with that used by Mattick & Hirsch (1947). It was propagated by daily subculturing in yeast dextrose lemco broth, in which medium nisin was also

readily formed. Stock cultures were kept in bullock's heart medium in the refrigerator.

Concentrates were made by a method similar to that of Mattick & Hirsch, viz. adsorption on to a $CHCl_3$ emulsion followed by removal of the $CHCl_3$. After growth the broth was acidified to pH 1·9±0·1, allowed to stand for 1 or 2 days and siphoned off. The pH was raised to 5·0 and 3% (v/v) $CHCl_3$ emulsified with the broth and kept suspended for about 30 min. After 2 hr. settling the supernatant liquid was siphoned off. The residue was acidified with 0·12 ml. of 10N-HCl/l. of original broth, the $CHCl_3$ distilled off *in vacuo* below 40° and the remaining suspension filtered at pH 1·5±0·1. The filtrate was boiled to destroy possible enzymes and concentrated fivefold *in vacuo*. An inactive precipitate was removed by raising the pH to 1·9±0·1 and adding 2 vol. of ethanol and 1 of ether; the nisin itself was precipitated with a further 3 vol. of ethanol and 5 of ether and dried with ethanol and ether. Powders so prepared usually had specific activities of about 1 unit/μg.

Fractionation in phosphate buffer. It was found that a product of seven or eight times the specific activity of the crude material could be prepared by treatment with M-K phosphate at pH 6·1, most of the activity remaining in the small insoluble portion. A solution of precipitate prepared in this way had a specific activity of 8 units/μg., and a dry powder with a specific activity of 8 units/μg. was obtained by precipitation with alcohol and ether.

The effect of pH on the fractionation was explored in an experiment in which 0·56 g. of crude powder (kindly supplied by Benger's Ltd.) was suspended in 12 ml. M-H_3PO_4, and precipitates were obtained and separated serially as the pH was raised from 1·4 through 2·3 and 3·8 to >6 and <7. After dissolving the precipitates in 0·05N-HCl their activities and organic dry weights were determined, giving the results shown in Table 1, from which it is clear that fractionation at the lower pH values gives less increase in purity than was obtained in the previous experiments.

Table 1. *Fractionation in molar potassium phosphates at various* pH *values*

	Total activity (units × 10^6)	Specific activity (units/μg.)
Original powder	0·6	1
Fraction:		
(1) Precipitate at pH 1·4	0·6	3·4
(2) Precipitating between pH 1·4 and 2·3	0·2	3·8
(3) Precipitating between pH 2·3 and 3·8	0·1	1·9
(4) Remaining soluble at pH 3·8 to >6 and <7	0·08	0·2
Recovered in fractions	0·98	

The increase in total activity shown in Table 1 contradicted the first four experiments in which the yields appeared to be very low. The gains occurring in the following detailed example of the fractionation at pH 6·1 in M-K phosphate were therefore determined as accurately as the method of activity measurement (*b*) would allow. Nisin powder (5·35 g., 0·9 unit/μg.) was dissolved in 106 ml. of 0·1N-HCl giving a turbid mixture of pH 0·9. The precipitate obtained on centrifuging dissolved readily in 0·05N-HCl and was added to the nisin residues. To the supernatant liquid sufficient KH_2PO_4 was added to bring the concentration to molar, i.e. 14 g., and after solution, the pH was raised to 6·11 with concentrated KOH (about 10 ml.). The precipitate, which began to form before the pH reached 3, increased until about pH 5, and decreased again greatly by pH 6. That which remained was allowed to stand until well flocculated, but even so the supernatant liquid obtained by centrifuging was not clear. A clear supernatant was produced, however, by a second centrifuging after the liquid had been allowed to stand overnight in the refrigerator. Thus a main and a subsidiary precipitate were obtained. The main precipitate was dissolved in 30 ml. of distilled water and adjusted with HCl to a final pH of 1·9. The solution had an activity of 250,000 units/ml. The total activities existing at the various stages are recorded in Table 2.

Table 2. *Increases in activity during fractionation in phosphate buffer*

		Total activity (units × 10^6)
Starting material (5·35 g. at 0·9 unit/μg.)		— 4·8
Solution in 0·1N-HCl (A)	4·6	6·2
Precipitate from 0·1N-HCl	1·6	
Solution (A) after adding phosphate and adjusting to pH 6·1 (sample 'activated' by standing overnight in 100 ml. 0·05N-HCl)		— 5·5
Solution of main precipitate	7·8	8·5
Solution of subsidiary precipitate	0·4	
Supernatant buffer solution (after acid 'activation' as above)	0·3	

In this experiment, therefore, $7·8 \times 10^6$ units, with a specific activity of presumably about 8 units/μg., were prepared from $4·8 \times 10^6$ units, with a specific activity of 0·9 unit/μg. This apparent increase has been confirmed but not further investigated.

Further purification by fractional precipitation with sodium chloride. An exploratory fractionation of the solution from the above experiment was made by dissolving in it gradually increasing quantities of NaCl, centrifuging as soon as a fifth to a tenth of the nisin appeared to be precipitated, and repeating the process until no further precipitate appeared. In this way five roughly equal fractions were obtained. Their specific activities were respectively 20, 18, 27, 24 and 5·0 units/μg. There seemed to be a loss of about 50%, but the activities of some of the acid solutions of the precipitates increased on keeping. Attempts to purify these fractions further by other methods, which are described below, were unsuccessful. Purification was therefore again attempted with fresh material.

Crude nisin powder was fractionated with phosphate buffer as already described, giving a solution of purified nisin in dilute HCl at pH 2. A slight precipitate produced by adding NaCl was discarded, and the bulk of the nisin then precipitated by saturation with NaCl and redissolved at pH 1·8. From 10 million units of crude nisin 130 ml. of purified solution containing 17 million units were obtained, an increase as before. Seven portions of NaCl were added in turn to this solution, the precipitate salted out by each being collected for the determination of total and specific activity. The procedure and results are summarized in Table 3. The final solution at pH 1·0 contained an insignificant quantity of nisin.

Table 3. *Fractionation of nisin solutions by precipitation with sodium chloride*

(Original solution 17×10^6 units in 130 ml. at pH 1·8, prepared by phosphate fractionation and preliminary precipitation with NaCl. NaCl (x g.) was added to supernatant number (Y) to give precipitate number ($Y+1$).)

			Resuspended precipitate ($Y+1$)		
Supernatant number (Y)	NaCl added (x g.)	Precipitate number ($Y+1$)	Specific activity (units/μg.)	Total activity (units $\times 10^6$)	pH
0	4·5	1	20	—	2·3
1	1	2	30	2·9	—
2	1	3	23	1·7	2·5
3	1·5	4	31	1·4	—
4	3	5	34	2·6	2·6
5	4·5	6	17	1·0	—
6	15	7	16	0·9	2·8
7	—	—	—	—	1·0

Thus, considerable increases in specific activity could be achieved by fractionation with NaCl.

Crystallization. Precipitates 2, 3 and 4 (selected before the specific activities had been determined) were united (20 ml. in all), adjusted to pH 2 and the solution saturated with NaCl. The resulting precipitate was washed with 3 ml. of distilled water, and then extracted three times with distilled water containing sufficient NaOH to give, in the mixture, a pH of 6·2, as described for extractions at pH 5·6 under 'Other methods of concentration and fractionation' (p. 491). Numerous attempts were made to crystallize these extracts by various techniques, especially by several different ways of slowly adding NaCl. The precipitates obtained were always granular and amorphous, but occasionally they contained a minute proportion of straight needles. The third of the extracts had been precipitated with NaCl several times at pH 2 when crystallization from ethanol was attempted. An equal volume of absolute ethanol was added to the centrifuged precipitate causing its immediate solution, followed by a slower precipitation which was reversible and dependent on temperature. On cooling the solution gradually from 37 to 0° a semi-solid suspension of long thin needles was formed. This was filtered in the refrigerator through a small disk of filter paper (about 1 cm. diam.), washed with a little ice-cold 80% (v/v) ethanol, and dissolved in 1 ml. of 0·05 N-HCl. Triplicate determinations of the organic dry weight of this solution gave 0·65, 0·61 and 0·63 mg./0·1 ml. The activity was 4600 units/0·1 ml. Thus the specific activity of the crystals or tactoids was 73 units/μg.

Further preparations of 'crystalline' nisin. Comparatively large quantities of 'crystalline' nisin were needed for animal experiments, and a series of eight preparations were next made from dry powders at 10 units/μg., which were now being produced by a small pilot plant.

The preparations were made according to modifications of the method described under 'Final preparation' (p. 490). In the first four of them particular attention was paid to the effect of various modifications on the yield. These yields, based not on weight but on activities, were respectively 24, 15, 26 and 15% of the starting material, but to avoid prolixity the experience of the whole series of preparations is summarized in the following points:

(1) It was thought that previous failures to crystallize from aqueous media might have been due to impurities which ethanol crystallization would remove, but the 'crystalline' substance, transferred to 0·05 N-HCl and salted out with 0·25 saturated $MgSO_4$ solution reappeared in the amorphous condition, although the $MgSO_4$ was added so slowly that 6 days were required for the precipitation.

(2) Most of the preparations contained a small quantity of gummy substance which would not dissolve in 80% ethanol but which could be removed by filtration at 37°, a process requiring about 24 hr. An attempt to increase the yield by increasing the middle fraction at the expense of both of the others led to such an increase in the quantity of the gummy substance that filtration was practically impossible. However, repeated extraction with 80% ethanol at 37° by centrifuging gave clear liquids which 'crystallized' well, but the yield was low.

(3) The separation of too little of the least soluble fraction led to a middle fraction which remained amorphous indefinitely during repeated 'recrystallizations'.

(4) Considerable quantities of nisin could be recovered from abortive experiments, and from mother liquors and other ethanolic residues by diluting with 5 vol. of distilled water, precipitating by saturation with NaCl and fractionating according to the simple unmodified scheme.

(5) Unless the properties of the starting material rendered the least soluble fraction inordinately large its further fractionation was uneconomic. 'Working up' processes of varying complexity, but always on the lines of the main preparation, were tried out on the least soluble fractions of three preparations. From only one was a significant increase in yield obtained.

(6) Nisin can be precipitated from dilute solution in 80% ethanol by raising the apparent pH to about 6, but whenever this was done there were great losses in activity.

(7) The correct acidity is essential for the formation of regular needles. Different quantities of HCl and NH_3 were added to samples of a suspension which had precipitated in the usual form of very long thin needles. The less acid suspensions needed considerable dilution before they could be brought into solution. After warming to dissolve and cooling slowly the precipitates were in the form of irregular thin crumpled plates from solutions of pH 2·42, 2·74 and 3·60, and microscopic needles at pH 4·22 diminishing again to microscopic threads at pH 4·74.

These observations suggest that the losses sometimes experienced as a result of difficulty in redissolving the needles in 80% (v/v) ethanol could have been avoided by using acidified ethanol and adjusting the pH after solution.

(8) The size of the needles could be considerably increased at the optimum acidity by controlling the rate of cooling. All arrangements of lagged vessels involve, at the

beginning of the process, a comparatively rapid cooling which leads to the formation of numerous small particles. With the apparatus already described (see Methods) this effect may be avoided without unduly prolonging the period of cooling. When cooled at the rate of 0·5°/hr. a sample which had previously given 'crystals' appearing under the microscope ($\frac{3}{4}$ in. objective) as fine needles now formed a precipitate recognizable as needles with some difficulty by the naked eye, but with ease at a magnification of 10 diameters.

(9) Several other methods of crystallization were tried without success, such as, for example, allowing an aqueous solution to evaporate in a desiccator at atmospheric pressure, removing water from a solution in 80% (v/v) ethanol by keeping in a desiccator over Ca metal (nisin is soluble in 80% but not in absolute ethanol), and stirring an acid ethanolic solution in a desiccator over dilute ammonia.

Final preparation. Nisin powder, 17·4 g. at 10 units/μg., was suspended in 700 ml. of distilled water by gentle stirring during about 30 min. The addition of 3 ml. of concentrated HCl (36% w/v) brought all but traces of the nisin into solution to produce a brown turbid liquid. After its pH had been brought up to 1·8 with a little 15% (w/v) ammonia, 28 g. NaCl were dissolved in the nisin solution and the resulting dark brown gelatinous precipitate filtered off immediately with Whatman no. 54 paper. Notwithstanding the increasing opalescence of the filtrate resulting from equilibrium not having been established, a further 4·4 g. NaCl/100 ml., actually 29 g. in 650 ml., were added. The precipitate which now formed was stirred in its mother liquor for about 30 min. before being filtered off (again no. 54 paper; both these filtrations were very rapid). The cream-coloured semi-solid precipitate was easily removed from the paper and was resuspended in enough distilled water to give a volume of 360 ml., in which it dissolved slowly. One half volume of saturated NaCl solution was now added slowly with continual stirring. By repeated gentle centrifuging the resulting precipitate was easily consolidated in one tube where it was dissolved by warming and shaking with about 1·2 vol. of absolute ethanol to give 150 ml. of clear brown solution. The 'pH' ('pH'=Glass electrode reading of 0·1 ml. ethanolic solution + 1·1 ml. distilled water) of this solution was raised with dilute ammonia from 3·5 to 4·21, a procedure which diminished the solubility of the nisin so that it was now necessary to warm it to 45° before complete solution occurred. On cooling from this temperature at the rate of 1°/hr. without stirring, comparatively large needles were formed. The brown colour remained in the mother

Table 4. *Purification of nisin*

Procedure	Quantity (units × 10^7)	Purity (units/μg.)
17·4 g. powder in 700 ml. + 28 g. NaCl	21	12
Precipitate (discarded)		
Filtrate	17	18
+ 29 g. NaCl		
Filtrate (discarded)		
Precipitate	12	24
Dissolved in water to 360 ml. + 180 ml. saturated NaCl solution. Centrifuged		
Supernatant (discarded)		
Precipitate	14	26
Dissolved in ethanol to 180 ml. at 45° and 'pH' 4·2. Cooled at 1°/hr. Centrifuged		
Mother liquor (to residues)		
Needles	11	40
Redissolved in 80% (v/v) ethanol at 37° and 'pH' 4·2. Filtered at 37° and recrystallized by slow cooling		

liquor. On resuspending the precipitate in 80% (w/v) ethanol a mixture of 'pH' 4·7 was obtained and some difficulty was encountered in achieving complete solution even at 'pH' 4·2. Although 80% (w/v) ethanol was added until the volume of suspension was 280–300 ml. some insoluble particles indistinguishable from the needles remained. (Probably better results would have been obtained by dissolving the precipitate at 'pH' 3·0–3·5 in a volume equal to that of the mother liquor and raising the 'pH' to 4·2.) The dilute solution was filtered overnight at 37° before sampling for yield and purity. On cooling at 0·5°/hr. it gave needles similar to the former ones but practically white in colour. They were smaller than those mentioned in (8) above. The process is summarized in Table 4. At this early stage it cannot be stated whether the discrepancy between the specific activity of this material and that of the 'crystals' first obtained arose from errors in the measurements of activity, from errors in standardization, or from actual differences in composition.

Other methods of concentration and fractionation. At pH values near neutrality crude nisin was adsorbed from the broth in which it had been produced on to each of all the insoluble substances tested, e.g. aluminium hydroxide, charcoal of all grades, benzoic acid (pH 3–4), emulsified ether, ethyl acetate, $CHCl_3$, CCl_4, trichloroethylene and cellulose (filter-paper pulp). It was always difficult to recover the nisin from the adsorption complex. Occasionally, as with cellulose, it could be obtained by extraction at pH 1·8, but even so the losses were high.

From solution at about 1000 units/ml. nisin was readily precipitated by a variety of protein-precipitating reagents such as tannic acid, phosphotungstic acid and uranyl acetate, but again the difficulty was the recovery of the material in an active condition.

Further fractionation of the concentrated, partly purified material (200,000 units/ml., 8 units/μg.) by partial precipitation of an ethanolic solution with ether gave some promise of success, but was eventually abandoned as unattractive.

Repeated extraction of precipitated nisin at pH 5·6 with distilled water gives results similar, but perhaps inferior, to those from partial precipitation with NaCl. In a preliminary experiment beginning with a preparation at 8 units/μg. it was possible to account for roughly 50% of the activity, while the last extract had a specific activity of 27 units/μg. A second experiment was made in which 4·4 g. of powder at 1 unit/μg. were subjected to fractionation in phosphate buffer (see p. 488), the nisin being precipitated from the final solution at pH 1·7 by saturation with NaCl. The precipitate was extracted by 'creaming' it with distilled water, diluting to about 20 ml. with distilled water, adjusting its pH to 5·5–5·7, and centrifuging. The procedure was repeated twice, using the remaining residue each time. Three extracts and one residue were thus obtained. The first extract only was further purified by centrifuging away the first turbidity which appeared when NaCl was added very gradually, precipitating the remaining nisin by saturation with NaCl, and extracting this precipitate once with distilled water at pH 5·5. All solutions were now precipitated by saturating with NaCl and centrifuged. These and the other precipitates were finally dissolved in 2–5 ml. 0·05 N-HCl before testing their activities. The results are shown in Table 5. Neither these nor those of Table 3 are adequate for a comparison of the methods, because the differences are not great. In the absence of further evidence fractionation with NaCl is, however, to be preferred on account of the greater stability of nisin at the lower pH values there employed.

Table 5. *Fractionation of nisin at* pH 5·6

	Quantity (units × 10^6)	Specific activity (units/μg.)
Original powder	4·4	1·0
Fractions:		
Phosphate fraction	—	8*
First extract:		
First turbidity with NaCl	0·07	—
Extract at pH 5·5	0·22	7·7
Precipitate at pH 5·5	0·26	—
Second extract	1·4	23
Third extract	0·79	23
Residue	0·71	—
Total recovery	3·45	

* Assumed on the basis of other experiments.

PROPERTIES OF NISIN

The biological properties of crude nisin preparations have already been described (Mattick & Hirsch, 1947; Hirsch, 1949).

Stability. Dry nisin powder appears to keep indefinitely in an ordinary atmosphere. At pH 6·8 and 37° in dextrose lemco broth nisin retains its activity for at least 24 hr.; solutions at pH 1·8 are stable for weeks. Boiling at pH 1·8–2·0 for 5–10 min. tends, if anything, to increase slightly the activity of dilute nisin solutions. Moreover, concentrated solutions prepared by dissolving 'crystals' from ethanolic solution in dilute HCl and adjusting to pH 2·0 may be boiled in an open beaker until free from ethanol without appreciable loss of activity, and saturated solutions at pH 4·2 may be 'sterilized' for injection by boiling for 10 min. Autoclaving, however (10 lb./sq.in. for 10 min.), destroys about 50% of the activity. In boiling 2 N-HCl destruction is complete after 2 min. In alkaline solutions the activity is quickly destroyed. An acid solution was immediately inactivated by potassium permanganate but not by sodium sulphide.

Nisin was suspected to be a peptone. It appeared possible therefore that it would be rapidly destroyed in an enzyme-rich medium such as blood. Preliminary experiments showed that nisin could be assayed in blood using method A i with an inoculum of 1% of a 24 hr. culture of *Strep. agalactiae*. Method B could also be used. It was then demonstrated by the former method that 6000 units/ml. in blood at 37° remained undiminished for at least 22 hr. Method B was then used to demonstrate an equal stability of nisin under similar conditions at a level of 15 units/ml. Fresh citrated whole blood was used.

Solubility. (i) *In blood.* As nisin was known to have a comparatively low solubility at pH levels near neutrality its solubility in blood became of interest as a factor which might limit its therapeutic use. After the preliminary tests described above, method B was used to measure, first, the distribution between corpuscles and plasma and, secondly, the activity of plasma after adding various quantities of nisin to blood and centrifuging. It was found that the nisin was distributed evenly between the plasma and the corpuscles, medium concentrations remaining unaffected by

centrifuging, and that the 'solubility' in blood is not less than 5000 units/ml., a value not too low to prevent its therapeutic use.

(ii) *In water.* At low pH values nisin is very soluble. At a pH about 4·2 saturated solutions of 'crystalline' nisin had an activity of 600,000 units/ml., corresponding to a solubility of approximately 1·5% (w/v). In extraction experiments nisin at about 20 units/μg. gave solutions containing 40,000 units/ml. at pH 5·6, but here adsorption effects may have interfered.

(iii) *In organic solvents.* Preliminary qualitative tests indicated that those compounds which dissolved nisin usually destroyed its activity, e.g. glacial acetic acid, benzyl alcohol. Ethanol (80% w/v) and formamide were exceptions, in that full activity was retained.

Molecular size. A rough measurement of the diffusion coefficient of nisin was made using the sintered disk method. This method was chosen because the results can be obtained from activity determinations alone, inactive impurities causing only indirect effects, such as, for example, the possible effect of rapidly diffusing molecules on the viscosity of the liquid into which a slower molecule might diffuse. It was, furthermore, impossible to find laboratory accommodation free from vibration. The details of the arrangement employed are described under Methods (p. 487).

A preliminary single determination of the diffusion coefficient gave 15×10^{-7} cm.^{2}/sec. In the next experiment diffusion was allowed to proceed for a total of 45·7 hr., the outer liquid being renewed at intervals. It is probable that the differences which still appeared (see Table 6) after equilibrium should have been established were due to uncertainties in the biological assay. Thus the value of 14×10^{-7} cm.^{2}/sec. for the diffusion coefficient of nisin is merely approximate. Nevertheless, it serves to show that nisin is probably a small protein or large polypeptide molecule (cf. D (diffusion coefficient at infinite dilution) $= 10{\cdot}57 \times 10^{-7}$ for lactalbumin, $10{\cdot}11 \times 10^{-7}$ for cytochrome *c*: Polson, 1939; Schmidt, 1943).

cipitated from ethanolic solution, was dissolved in 0·05 N-HCl and salted out by the slow addition of NaCl through a rotating membrane (McMeekin, 1939). The resulting granular precipitates were convenient for settling, filtering and washing. The material for the first experiment was equilibrated with buffer by five washings on a sintered glass filter, that for the second by dialysis with renewal of buffer daily for 13 days. After this second procedure, 25% of the nisin was discovered to be insoluble; probably chemicals from the insufficiently washed collodion membrane brought

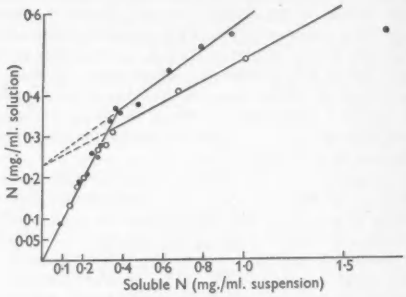

Fig. 1. Solubility of nisin: ●, Exp. 1; ○, Exp. 2.

about denaturation; 25% was therefore subtracted from the figures for total N in the second solubility experiment. The buffer was made by dissolving 26 g. KH_2PO_4 and 60 g. KCl in 800 ml. of distilled water, adding 10 N-HCl to pH 1·92 and finally making up to 1 l. Nisin suspensions of different concentrations were made, and after equilibrium had been established the solutions were separated and analysed for nitrogen as already described (see p. 487). Both sets of results are shown graphically in Fig. 1, from which it is clear that these preparations contained at least two components,

Table 6. *Diffusion of nisin*

(Inner solution; nisin, 65 ml. at 175,000 units/ml. Outer solution; solvent, 25–50 ml.)

Diffusion no.	Time during which diffusion occurred (hr.)	Units diffused	Units diffused/hr.		Diffusion coefficient (cm.^{2}/sec.)
1	4·5	81,000	18,000		—
2	17·1	94,000	5,500		
3	12·0	80,000	6,700	Mean **6,400**	14×10^{-7}
4	12·1	85,000	7,000		

Amino-acid composition. Paper partition chromatography (Consden, Gordon & Martin, 1944) was used to analyse a hydrolysate of 'crystalline' nisin. The following amino-acids were recognized; alanine, valine, leucine, isoleucine. Cystine and aspartic acid were possibly present. There were also several faint or unrecognizable spots.

Purity. The solubility of nisin depends on the quantity of solid phase present; when the concentration of the solution was plotted against that of the suspension two straight lines of different slope were obtained showing that the preparation contained at least two components. A solution saturated with respect to all components was not obtained.

Two main experiments were carried out. For each of them a different preparation of nisin, in the form of needles pre-

the less soluble of which amounted to about two-thirds of the total mixture. The possibility mentioned by Butler (1941) that different forms of one component may persist although not in equilibrium, remains to be examined, but it seems remote because of the slow precipitation of the solid used in these experiments.

DISCUSSION

Throughout the work there were a number of unexplained observations which could not then be further investigated. The increases in activity recorded in Tables 1 and 2 are among them. Some

of the others may be merely mentioned, for example: (*a*) the activity of acidified broth containing nisin was destroyed on attempting to concentrate *in vacuo*, some activity reappearing on keeping; (*b*) when the organism was allowed to grow at 22° instead of 30° a concentrated product active against *Strep. cremoris* but not against *Strep. agalactiae* was obtained; (*c*) significant yields were obtained in some media after steaming, but not after autoclaving, although the organisms still grew well; and (*d*) the sensitivity of nisin to certain chemicals and solvents contrasts strangely with its stability to others and to acids and heat.

It was impossible to prove that the needles observed were true crystals. Their shape in cross-section could not be observed. The slow filtering and the slightly syrupy nature of the ethanolic solutions, as well as the formation of gels when such solutions were rapidly cooled, suggests that the needles may well have been tactoids.

Although a few preliminary experiments have shown that nisin is closely associated with a polypeptide, the solubility measurements indicate that further purification is required before the properties of the active substance, or substances, can be properly investigated. Meanwhile, the purification so far achieved has facilitated experiments in other directions.

SUMMARY

1. Experiments in concentrating and purifying the antibiotic nisin are described.

2. Nisin may be obtained in the form of needles by cooling a solution in 80% ethanol at 'pH' 4·2 ('pH' is glass electrode reading of ethanolic solution diluted with 11 volumes distilled water).

3. The properties of the 'crystalline' product have been investigated. It will inhibit the growth of the more sensitive organisms at dilutions from 10^{-8} (*Strep. agalactiae*) to 10^{-9} (*Strep. cremoris*); it is stable for weeks in acid solution and for many hours in blood at 37°; it contains the amino-acids alanine, valine, leucine, isoleucine and others, and appears to consist of a mixture of at least two polypeptides.

Special thanks are due to Benger's Ltd. (of British Chemicals and Biologicals Ltd.) for their co-operation in making, and generosity in supplying, active concentrates. The helpful encouragement given by the Director, Prof. H. D. Kay, and the invaluable support of the Assistant-Director, Dr A. T. R. Mattick, are gratefully acknowledged. Thanks are also expressed to Dr A. Hirsch for useful suggestions. The work described under 'Preparation of crude concentrates' was carried out in the laboratories of Benger's Ltd., whose Director, Mr B. D. Thornley, is to be thanked, as are also Dr E. G. Stopher, Mr C. Epstein, and Miss E. M. Barnes, members of the staff, for their assistance. The later help of Mrs C. Waterhouse is acknowledged with thanks.

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The Cholinesterase Activity of the Serum of Newborn Animals, and of Colostrum

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The activities of the enzymes in serum which split acetylcholine have been investigated fairly extensively in man, both in health and disease. Enough work has also been carried out on animal sera to establish the general level of enzyme activities in the various species, and active preparations of the so-called 'pseudo' enzyme (Mendel, Mundell & Rudney, 1943) have been made from various tissues. It is, however, believed that no tests have hitherto been made on newborn animals, or on the colostrum and milk of their mothers. Human beings, rats, rabbits, guinea pigs, cats and dogs have been investigated in the work to be described.

METHODS

The adult humans were healthy males. Blood was taken from them by vein puncture with a minimum of stasis. The infants were of both sexes and were all full term and normal. Their mothers were also normal and had given birth to the children without assistance or anaesthetic. Blood was taken from infants at birth by puncturing the umbilical cord with a large-bore needle or by allowing the maternal end of the cord to drain into a centrifuge tube, and later from the heel by cutting out a disk of skin with a scleral trephine and allowing the blood to flow without pressure into a small tube. Blood was obtained without anaesthesia from adult animals by vein puncture or decapitation, and from young animals by heart puncture from the following species: rats (Lister Institute strain); guinea pigs and rabbits (Department of Pathology stock); cats and dogs of various breeds. At birth and during the first 7 days thereafter, blood had to be withdrawn from several rats to get enough serum for each determination. After the samples were taken, the serum was separated by centrifuging as soon as possible, with care to minimize evaporation. Puncturing the heart did not appear to harm the young animals, and, after a few days, second and even third samples of blood were sometimes taken from the same animal by this method. Colostrum and milk were obtained by manual expression.

The measurements of enzyme activity were made manometrically at 37° (McArdle, 1940), using 1 ml. of 2·5% acetylcholine chloride as substrate in a total volume of 3 ml. of fluid. The liberation of 1 μl. of CO_2/min./ml. serum, colostrum or milk has been defined as the unit activity.

RESULTS

Man. The average activity of 28 adult sera was 76 units (S.D. 13·8), which agreed very well with the figure obtained by McArdle (1940). The mean activity of 39 cord sera was 50 units (S.D. 10·7). This difference was significant ($t = 12{\cdot}50$; $P = < 0{\cdot}01$). The changes in activity following birth are shown in Table 1.

Table 1. *The cholinesterase activity of the sera of newborn infants*

Age	No. of samples	Average activity (μl. CO_2/ml./min.)	Standard deviation
At birth	39	50	10·7
0–24 hr.	39	62	14·1
5–10 days	71	64	15·7
11–21 days	19	75	14·3
Over 3 weeks	4	87	2·7

The apparent rise in the first 24 hr. was checked on numerous occasions by taking both specimens of blood from the same baby. The increase in the average activity of the sera of the 39 babies from 50 to 62 units was statistically valid ($t = 6{\cdot}93$; $P = < 0{\cdot}01$). This rise, however, may not represent a real change in enzyme activity, since the two specimens of blood were not taken from the same site, and peripheral blood has been shown to be more concentrated, or at any rate to carry more haemoglobin/ml., than cord or sinus blood (DeMarsh, Alt, Windle & Hillis, 1941; DeMarsh, Alt & Windle, 1948). These observations have been confirmed. The subsequent rise of enzyme activity was to be expected at some period of early childhood, since McArdle (1940) found older children to have higher serum activities than adults and this has been confirmed by one of the authors (Hutchinson, 1949). No relationship has been found between the level of activity in cord sera and the weight of the child at birth, or the length of its gestation period as reckoned from the beginning of the mother's last menstrual period. No correlation has been found, moreover, between the enzyme activity of cord sera and the mother's age or her clinical status. The level of enzyme activity of colostrum, and milk up to the 22nd day, has been found to be negligible. Many specimens had no measurable activity and only one specimen out of 30 had an activity as high as 10 units.

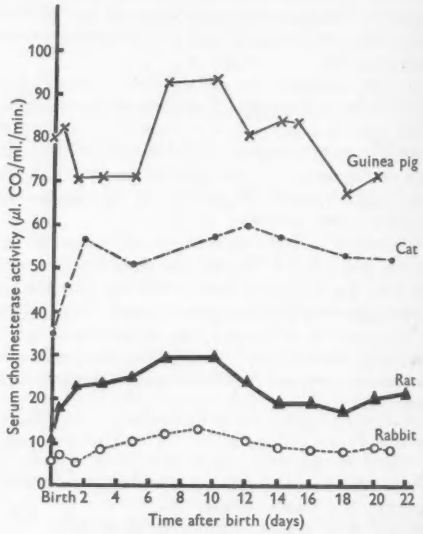

Fig. 1. Cholinesterase activities in the sera of young animals.

Rats, rabbits, cats and guinea pigs. Adult and newborn animals of these species have been investigated and the variations in the first 3 weeks of life are shown in Fig. 1. Several litters were used for the young of each species, and the points represent the mean of up to 8 specimens.

The following values had been found for the adult animals: rats, 13·1 units (mean of 6 males); rabbits, 12·2 and 11·5 units (2 males); cat, 24·0 units (1 female); guinea pigs, 40·0 and 57·0 units (2 females). The levels at birth were lower than those of the

adults in the rat and rabbit, and higher in the cat and guinea pig. Except in the guinea pig the general trend after birth was similar to that in man, in that there was a definite rise. The trend with the guinea pig was less certain.

The colostrum of one cat was investigated and found to contain less than 1 unit/ml.

Dogs. The sera of 6 normal adults, 4 of whom were males, and 2 non-pregnant females, averaged 38 units; and experiments have been made on 5 litters of puppies. The sera of puppies at birth had an activity of the same order of magnitude as the adults, but there was a rise in the first few days of life to about 800 units, and a fall in the next 2 weeks to about 50 units. Colostrum obtained from a bitch at the time of parturition was found to have an activity of 6000 units, and this activity declined rapidly at first and then more slowly. The milk of one animal had an activity of 54 units, and of another 400 units, by the 5th day, and was still 20–30 units after a fortnight. Composite curves of the findings plotted on a logarithmic scale are shown in Fig. 2.

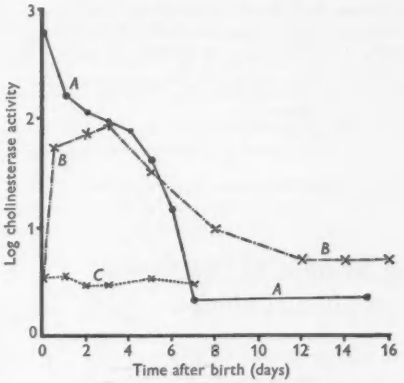

Fig. 2. The cholinesterase activities of puppy sera and of bitches' milk during the first 16 days after birth. *A*, activity of mother's colostrum and milk; *B*, activity in the sera of puppies feeding from the mother; *C*, activity in the sera of puppies fed on evaporated cow's milk.

The colostrum or milk expressible from glands which were not being suckled remained high after the activity in the milk from other glands had fallen to a lower level. Thus the activity of a pooled sample of fluid from unused glands on the 5th day was 918 units and on the 15th day was 2000 units.

The rise in the serum activity of the puppies depended upon the consumption of the mother's colostrum, for some puppies, which did not gain weight and thrive, presumably because they had not taken sufficient milk, had much smaller increases of activity in their sera than those which took milk freely from birth and gained weight rapidly. Furthermore, puppies reared on evaporated cow's milk, which contained no measurable cholinesterase activity, showed no increase in serum activity although they grew as well (and their eyes opened at the same time) as litter mates kept with the mother. This is illustrated in Fig. 2.

The activity of the bitches' serum did not rise very much post-partum although there may have been a small increase in some instances. In one animal, the serum, before delivery, had an activity of 47 units. This had risen to 74 units on the following day and remained above 60 units for at least 17 days.

The enzyme present in bitches' milk hydrolysed benzoylcholine and acetylcholine but not acetyl-β-methylcholine. It therefore corresponds to the 'pseudo-cholinesterase' of Mendel *et al.* (1943).

DISCUSSION

Until more is known of the source and function of the unspecific cholinesterase found in serum it is difficult to assess the importance of these results. It must remain for a time, therefore, an observation of fact that the activity of the sera of infants at birth tends to be below that of adults and to rise soon afterwards to levels comparable with those found in later childhood. A rise after birth seems to take place also in the rat, rabbit and cat, but the level at birth may be below or above that of the adults. Newborn guinea pigs had a higher level of activity in their sera than was found in the mature animals investigated and it did not rise after birth, and this may be connected with the advanced stage of development of this species at birth.

The dog must be considered separately for it shows a remarkable species difference in biochemical behaviour in that the colostrum contains a relatively enormous amount of cholinesterase, and it would be interesting to know at what stage of evolution this difference arose and whether it is exhibited by any allied genera and species. The evidence indicates that the source of the high activity in the puppies' sera was the colostrum of the bitch, and it seems, therefore, that the enzyme must have escaped digestion and passed through the wall of the intestine unchanged. The passage of proteins and immune bodies through the wall of the gut has been demonstrated in the young of some animals and suspected in others (Schloss & Anderson, 1922; Anderson & Schloss, 1923; du Bois, Schloss & Anderson, 1925), but, so far as we know, this is the first evidence for the passage of an enzyme. Cholinesterase is not thought to have any immunological significance nor is it known whether it plays any functional role in the mammary gland of the bitch or in the serum of a newborn puppy. Its lipolytic activity in the gut may be of value in the newborn

puppy but would not appear to be essential since the three puppies reared on evaporated cow's milk throve satisfactorily without it. Apart from this, however, bitches' colostrum should provide a valuable source of unspecific cholinesterase in high concentration, and it is hoped to investigate some of the problems arising out of the observations here presented.

SUMMARY

1. In newborn humans, rats, rabbits and cats the cholinesterase activity of the serum tended to be near the adult level and to rise soon afterwards to about twice the initial value. In guinea pigs the activity did not rise.

2. The colostrum of man and of the cat had negligible cholinesterase activity.

3. In puppies the serum cholinesterase rose to 25 times its birth level in 3 days. This increase appears to be due to the very high cholinesterase activity of bitches' colostrum.

The human material was obtained from the Landesfrauenklinik, Wuppertal, through the courtesy of the Director, Prof. Anselmino. A pure-bred setter was obtained from Mr Parry, the Canine Research Station, Kennet. Our thanks are also expressed to Miss M. F. Harrison for considerable assistance with work carried out at night.

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The Glucuronide-synthesizing System in the Mouse and its Relationship to β-Glucuronidase

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The β-glucuronidase activity of mouse liver, kidney or uterus has been shown to reflect the state of proliferation of the tissue (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949*a*). It would appear that β-glucuronidase is distinct from the enzyme system responsible for the production of conjugated glucuronides in the body. The work of Lipschitz & Bueding (1939) suggests that this process is more complex than a simple condensation of the aglycone with free glucuronic acid, and that it takes place in liver and kidney only. β-Glucuronidase, on the other hand, is present to a greater or less extent in practically all animal tissues that have so far been examined (see, for example, Oshima, 1934). Karunairatnam & Levvy (1949) found that glucuronide synthesis by adult mouse-liver slices was not appreciably impaired by saccharic acid in concentrations which caused almost complete inhibition of β-glucuronidase.

The ability of various mouse tissues to synthesize glucuronides has been studied and compared with their glucuronidase activity under conditions leading to changes in the latter.

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EXPERIMENTAL

Measurement of glucuronide synthesis. The conversion of *o*-aminophenol to its glucuronide in sulphate-free bicarbonate Ringer solution was followed by the method of Levvy & Storey (1949). Except in the case of lung, the tissue was sliced, and an amount corresponding to not less than 10 mg. dry weight was taken for each estimation. If necessary, slices from more than one animal were pooled. Whenever possible, the estimation was done in quadruplicate in order to reduce the variable error in the procedure (see Levvy & Storey, 1949). In the case of lung, the intact lobes were used. Boyland & McDonald (1948) have shown that this is permissible for measurements of metabolism in lung from young adult mice.

Results are expressed in the tables as μg. *o*-aminophenol conjugated/g. dry weight of tissue in 1 hr. Both male and female mice were studied, but sex did not have any apparent effect on values for glucuronide synthesis, and it is therefore not usually shown in the tables.

Glucuronidase activity. The activity of this enzyme in tissue extracts was determined by the method of Kerr, Graham & Levvy (1948), and results are shown in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015 M-phenylglucuronide at 37° and pH 5·2. In the case of tissues in which the kinetics of hydrolysis of phenylglucuronide by the enzyme have not yet been studied, it was assumed that optimal conditions for hydrolysis resemble those found for liver, spleen and kidney (Kerr *et al.* 1948; Kerr *et al.* 1949*a*).

RESULTS

Comparison of glucuronide synthesis and glucuronidase activity in various tissues. Table 1 shows the glucuronide-synthesizing power of liver, kidney, spleen and lung in young and adult mice, and of two transplantable mouse tumours. In agreement with Lipschitz & Bueding (1939), the synthetic system was found only in liver and kidney, the latter being much the less active of the two tissues. The ability of kidney slices to synthesize glucuronides was considerably less in young mice than in adults. This was also true for liver, and Fig. 1 shows the development of the synthetic system in the liver of the growing mouse.

Table 1. *The glucuronide-synthesizing power and β-glucuronidase activity of various mouse tissues*

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Tissue	Age of animal	*o*-Aminophenol conjugated (μg./g. dry weight)	Glucuronidase activity/g. moist weight (G.U.)
Liver	Adult	570±43 (41)	273±13 (23)*
Kidney	Adult	150±14 (4)	363±24 (11)*
	9 days	30 (4)	—
	5 days	—	793 (4)*
Lung	Adult	Nil (2)	185±19 (3)
	9 days	Nil (3)	316±22 (3)
Spleen	Adult	Nil (3)	636±70 (23)*
	9 days	Nil (4)	—
	5 days	—	3245 (4)*
Sarcoma (Crocker 180)	Adult	Nil (2)	433±44 (6)
Carcinoma (Imperial Cancer Research Fund 2146)	Adult	Nil (2)	751±75 (6)

* Quoted from Levvy *et al.* 1948.

Also shown in Table 1 are figures for the glucuronidase activity of the various tissues, some quoted from earlier work from this Department, and others especially determined for present purposes. The distribution of this enzyme obviously bears no relation to that of the synthetic system. The higher glucuronidase activity of organs from young mice compared with those from normal adults, already noted for liver, kidney, spleen and uterus (Levvy *et al.* 1948; Kerr *et al.* 1949*a*), is also seen in lung. The change in liver glucuronidase activity has now been fully studied from birth until maturity, and the results are shown in Fig. 2. This graph was compiled from results collected over a considerable period of time with four different strains of mice. The glucuronidase activity of the young mice at a given age was remarkably constant for the different strains, and average figures for adults of different strains were indistinguishable from each other.

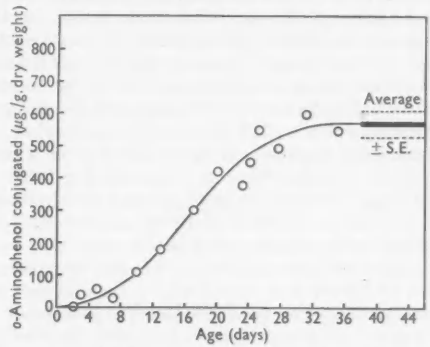

Fig. 1. The development of the glucuronide-synthesizing system, as measured by the conjugation of *o*-aminophenol, in the liver of the growing mouse (own mixed colony).

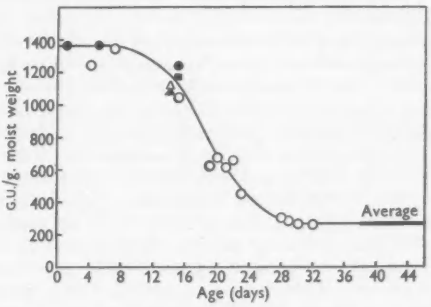

Fig. 2. The change in glucuronidase activity in the liver of the growing mouse: ■, strain C57; △, strain A; ▲, strain CBA; ●, own mixed colony, August 1947; ○, own mixed colony, February 1949.

In agreement with the observations of Fishman & Anlyan (1947) on human tissues, both mouse tumours were high in glucuronidase activity when compared with most normal adult tissues.

The role of glucuronidase in glucuronide synthesis. The possibility had to be considered that the activity of the glucuronide-synthesizing system in the liver does not vary with age, but that the hydrolytic activity of β-glucuronidase at any given age determines the net amount of synthesis which can be measured (compare Figs. 1 and 2). It has already been shown (Karunairatnam & Levvy, 1949) that saccharate in a concentration of 10^{-2}M causes almost complete inhibition of glucuronidase in liver or kidney extracts, but has no appreciable effect on glucuronide synthesis by adult mouse-liver slices. In many of the experiments summarized in Table 1, additional determinations of glucuronide synthesis were made in presence of 10^{-2}M-saccharate. This substance caused no increase in the synthetic power of infant liver or adult kidney, nor did its presence lead to glucuronide synthesis by lung, spleen or tumour slices. Failure of saccharate to penetrate the cell would appear to be excluded, since Campbell & Levvy (1949) have found that it inhibits hydrolysis of glucuronides by mouse kidney in the two histochemical tests of Friedenwald & Becker (1948). It seems, therefore, that the glucuronidase activity of a tissue has no bearing on its ability to synthesize glucuronides.

The effect of various measures on the glucuronide-synthesizing system in vivo. Various measures leading to changes in the state of proliferation of mouse liver were examined for their action on the glucuronide-synthesizing system. The results are shown in Table 2, and it can be seen that in no instance was there a detectable change in the activity of the synthetic system. Under similar conditions, a rise in glucuronidase activity to two or three times the normal value was seen after partial hepatectomy, or injection of menthol, carbon tetrachloride or oestrone (Levvy *et al.* 1948; Kerr *et al.* 1949*a*). Colchicine in the dose shown causes no change in the normal glucuronidase activity of liver, but it does prevent the rise in activity which follows such measures as partial hepatectomy. In the smaller dose, sorbic acid behaves like colchicine, but the larger dose (240 mg./kg.) causes a profound depression in liver and kidney glucuronidase activity in normal mice. The effects of colchicine and sorbic acid on glucuronidase activity appear to be related to their actions as inhibitors of mitosis (Kerr, Campbell & Levvy, 1949*b*).

The effect of various compounds on the synthesis of o-aminophenylglucuronide in vitro. Four of the compounds examined for their action on the glucuronide-synthesizing system *in vivo* were tested *in vitro* for their effect on the conversion of *o*-aminophenol (0·00023M) to its glucuronide by surviving liver slices from normal mice (Table 3). The fifth compound listed in Table 2, oestrone, was too sparingly soluble in water to permit its study in the present experiments.

Table 2. *The effect of various measures on the glucuronide-synthesizing system in mouse liver*

(When the mean is based on values for individual animals the standard error of the mean for the group is also shown. Figures in brackets are numbers of animals used.)

Treatment	Dose (g./kg.)	Days after treatment	*o*-Aminophenol conjugated/g. dry weight (μg.)
None	—	—	570±43 (41)
(−)-Menthol, intraperitoneally in olive oil	0·33	1	560±40 (3)
		3	640 (2)
		6	650 (2)
Carbon tetrachloride, subcutaneously in olive oil	5·33	1	560±170 (3)
		3	640±200 (3)
		7	640±150 (3)
Partial hepatectomy	—	3	670±60 (6)
		7	650±50 (6)
		10	610±80 (6)
Oestrone, subcutaneously in olive oil (ovariectomized mice)	0·0017	4	580±70 (3)
Colchicine, subcutaneously in aqueous solution	0·0015	1	450±80 (6)
Sorbic acid, subcutaneously in aqueous solution	0·24	4	440±70 (12)
	0·16	4	540±120 (6)

Table 3. *The effect of various compounds on the synthesis of o-aminophenylglucuronide by mouse-liver slices*

Compound	Concentration (M)	*o*-Aminophenol conjugated (μg./g. dry weight) In controls	In presence of compound	Inhibition (%)
Sorbic acid	0·01	380	50	87
	0·01	970	370	62
	0·005	970	500	49
Colchicine	0·01	380	140	63
	0·01	530	190	64
	0·005	530	270	49
Carbon tetrachloride	0·0015	490	520	−6
	0·0015	660	520	21
(−)-Menthol	0·001	450	120	73

Colchicine, sorbic acid and menthol were added as aqueous solutions (if necessary, after pH adjustment) during the preparation of the bicarbonate Ringer solution. In the case of CCl_4, the Ringer solution was made saturated with the compound. In all experiments, controls were done with untreated slices from the same animal. Each determination was done in quadruplicate, and the standard error of the mean is thus about 10% (Levvy & Storey, 1949).

As can be seen from Table 3, in the presence of colchicine, sorbic acid or menthol there was a drop in the conversion of *o*-aminophenol to its glucuronide which was outside the range of error. Inhibition was almost 50% with 0·005M-colchicine or sorbic acid, and 73% with 0·001M-menthol. Carbon tetrachloride had no appreciable effect in the highest concentration possible (0·0015M). None of the four compounds in question interfered in the colour reaction for *o*-aminophenylglucuronide.

DISCUSSION

From the results of the experiments described above, it seems clear that there are at least two distinct enzyme systems in the mouse concerned with the metabolism of the conjugated glucuronides. One of these, β-glucuronidase, is present in practically every tissue, and its action is probably entirely hydrolytic. The activity of this enzyme in a tissue varies with the degree of cell division in progress. The other enzyme system is responsible for the synthesis of glucuronides, and is probably complex. It has so far been found only in liver and, to a smaller extent, kidney. The activity of this enzyme system in liver is not altered by measures causing changes in the state of proliferation of the tissue. The ability of liver or kidney to synthesize glucuronides does, however, vary with the age of the animal. At birth, the activity of the synthetic system is small or nil, and it only reaches its ultimate value when the animals are 4 or 5 weeks old. Taken together, the two enzyme systems may provide a mechanism for regulating the transport, action and excretion of physiologically active, glucuronidogenic compounds, such as oestriol. Alternatively, their function may be to provide free glucuronic acid or a transformation product for building up into more complex molecules.

Since menthol is known to form a glucuronide in the presence of surviving liver slices (Lipschitz & Bueding, 1939), its depressant action on the synthesis of *o*-aminophenylglucuronide *in vitro* is probably the result of competition with *o*-aminophenol rather than genuine inhibition of the synthetic mechanism. The inhibitory actions of sorbic acid and colchicine on glucuronide synthesis *in vitro* are difficult to interpret at present. The overall synthetic process is known to be adversely affected by other agents, such as cyanide, fluoride and iodoacetate (Lipschitz & Bueding, 1939), azide and sulphate (Dr I. D. E. Storey, private communication), but their mode of action is in most cases still obscure.

SUMMARY

1. The glucuronide-synthesizing system in the mouse was found only in liver and, to a smaller extent, kidney.

2. Measures causing changes in the state of proliferation of liver had no effect on the activity of the glucuronide-synthesizing system.

3. The activity of the synthetic system was almost nil at birth, and only reached its ultimate value when the mice were more than 1 month old.

4. The effects of various compounds on glucuronide synthesis *in vitro* were studied.

5. On the basis of this work, the enzyme system responsible for glucuronide synthesis can be clearly distinguished from β-glucuronidase.

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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

1. LIPIDS

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When a peripheral nerve is cut, that portion of the nerve distal to the point of section soon loses its ability to transmit a nerve impulse. This is associated with a series of familiar histological changes known as Wallerian degeneration. Both histologists and histochemists have studied degenerating nerve in great detail, but in few instances have the techniques of chemistry been employed. Notable exceptions are the classical publications of Noll (1899), who observed that degenerating horse and dog nerves contained less 'protagon', and Mott & Halliburton (1901*a*, *b*), who reported the complete disappearance of phosphorus from degenerating cat nerves. These results, although of great interest, are of limited value because of the technical methods employed; usually a large sample of nerve tissue was needed and only one constituent of the nerve could be estimated from each sample. More recently, May (1930) has reported changes in the distribution of phosphorus and Abercrombie & Johnson (1946*b*) have described changes in the distribution of nitrogen, including collagen nitrogen, in degenerating rabbit nerves.

As the nerve degenerates, the lipid-containing myelin sheath that surrounds the axon of each individual nerve fibre at first fragments and later is completely destroyed. Although the principal constituent of this sheath, 'myelin', is usually referred to as though it were a chemical entity, chemists have for a long time suspected it to be a mixture of substances. Recently, Johnson, McNabb & Rossiter (1948*a*, *b*; 1949*a*) have produced evidence that free cholesterol and the two sphingosine-containing lipids or sphingolipids (cerebroside and sphingomyelin), rather than lecithin or kephalin, are the principal lipid components of the myelin sheath. They have called these lipids, i.e. free cholesterol, cerebroside and sphingomyelin, the myelin lipids.

For references to the extensive literature on the histological changes that occur during Wallerian degeneration the reader is referred to the publications of Ramon y Cajal (1928), Nageotte (1932), Weddell & Glees (1941), Young (1942), Holmes & Young (1942) and Weiss (1944). Since destruction of the myelin sheath is such an outstanding feature, it seemed of interest to investigate the changes in the concentration of the myelin lipids in a degenerating nerve. Micromethods are now available that permit the determination of these and other lipids in small samples of nerve. A preliminary account of these experiments has already appeared (Johnson, McNabb & Rossiter, 1949*b*).

METHODS

The right sciatic nerve of 30 cats was cut at the level of the greater trochanter of the femur. The proximal stump was retracted and sutured to the overlying muscle. By separating the proximal and distal stumps in this way the possibility of regeneration was minimized. None of the animals showed either functional or post-mortem evidence of regeneration. The operation was performed with full aseptic precautions under nembutal anaesthesia. No attempt was made to control the age, weight, or sex of the animals. After periods of time varying from 4 to 96 days the animals were killed and the distal degenerating segment of nerve removed. At the same time a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue and the lipids extracted with ethanol and ether as described previously (Johnson *et al.* 1948*a*). Samples of the ethanol-ether extract were placed in a 60° water bath and evaporated just to dryness under reduced pressure and in an atmosphere of N_2. The lipids were extracted with light petroleum (b.p. 40–60°) and the phospholipins precipitated with acetone and $MgCl_2$. The precipitate was washed with acetone and then dissolved in a 1:1 methanol-ether mixture.

The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin, total fatty acid and neutral fat was determined in each nerve. From these figures the concentration of ester cholesterol, sphingomyelin, kephalin, essential lipid (i.e. all non-triglyceride lipids), myelin lipid, and total lipid was calculated. All analyses were done in duplicate.

Analytical procedures

The analytical methods for cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin and lecithin have been described by Johnson *et al.* (1948*a*).

Cerebroside (glycosphingoside) was estimated in a sample of the original ethanol-ether extract by the method of Brand & Sperry (1941) in which galactose, liberated by acid hydrolysis, is estimated by the procedure of Miller & Van Slyke (1936). In this method, ferrocyanide, formed from the reduction of ferricyanide by the galactose, is titrated with ceric sulphate. Cerebroside was calculated as galactose $\times 4{\cdot}5$.

Total and free cholesterol were estimated in the acetone-soluble fraction by the method of Schoenheimer & Sperry (1934), incorporating improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945).

Total phospholipin. Total P was determined in a sample of the methanol-ether solution of the acetone-precipitated phospholipin by the method of King (1932). Total phospholipin was calculated as lipid P $\times 25$.

Monoaminophospholipin (phosphoglyceride). A sample of the methanol-ether solution of the acetone-precipitated phospholipins was hydrolysed in N-KOH for 18 hr. at 37°. Monoaminophospholipin was estimated as the total acid-soluble P of the hydrolysate (Schmidt, Benotti, Hershman & Thannhauser, 1946).

Lecithin (phosphatidyl choline) was estimated as the acid-soluble choline, determined by the method of Glick (1944), in the monoaminophospholipin hydrolysate (Hack, 1947). Lecithin was calculated as choline chloride $\times 5{\cdot}56$.

Total fatty acid was determined in a sample of the original ethanol-ether extract by a modification of the Bloor oxidative method described by Boyd (1938). After saponification and acidification, the free fatty acid and cholesterol were extracted with light petroleum (b.p. 40–60°) and oxidized by a measured quantity of $K_2Cr_2O_7$, excess of which was titrated with thiosulphate. After correction had been made for the cholesterol present, the total fatty acid was calculated on the assumption that 1 mg. of fatty acid was oxidized by 3·61 ml. 0·100N-$K_2Cr_2O_7$ (Boyd, 1938).

Neutral fat. An estimate of triglyceride was obtained by determining the glycerol in a portion of the phospholipin-free acetone solution by the method of Voris, Ellis & Maynard (1940). After saponification and acidification, the fatty acids and cholesterol were removed by extraction with light petroleum (b.p. 40–60°) and the glycerol allowed to react with a measured quantity of potassium periodate. Excess periodate was titrated with thiosulphate. The periodate used gave a measure of the glycerol in the sample. Neutral fat, based on an assumed mean molecular weight of 283 for the fatty acids, was calculated as glycerol $\times 9{\cdot}64$.

In addition, a value for neutral fat was derived from the figures for neutral-fat fatty acid, i.e. the difference between the total fatty acid and the sum of the fatty acids of cerebroside, cholesterol ester, lecithin, sphingomyelin and kephalin. In the absence of detailed information concerning the nature of the fatty acids of the lipids of nerve,* the figures were calculated on the basis of an assumed mean molecular weight of 368, that of lignoceric acid, for the fatty acids of the two sphingolipids, cerebroside and sphingomyelin, and an assumed mean molecular weight of 283 for the fatty acids of the phosphoglycerides and cholesterol ester. A molecular weight differing from the assumed mean for the fatty acids of either the sphingolipids or the phosphoglycerides would not appreciably alter the general conclusions. The neutral-fat fatty acids were, therefore, calculated as: total fatty acid (cerebroside $\times 0{\cdot}45$ + ester cholesterol $\times 0{\cdot}73$ + lecithin $\times 0{\cdot}70$ + sphingomyelin $\times 0{\cdot}44$ + kephalin $\times 0{\cdot}74$). Based on an assumed mean of 283 for the triglyceride fatty acids, the neutral fat was calculated from the neutral-fat fatty acid by multiplying by the factor 1·045.

Ester cholesterol was calculated as the difference between the total cholesterol and the free cholesterol.

Sphingomyelin (phosphosphingoside) was calculated as the difference between the total phospholipin and the monoaminophospholipin.

Kephalin, representing all the non-choline-containing monoaminophospholipins, was calculated as the difference between the monoaminophospholipin and lecithin.

Essential lipid, representing the non-triglyceride lipid, was the sum of cerebroside, total cholesterol and total phospholipin.

Myelin lipid was the sum of cerebroside, free cholesterol and sphingomyelin.

Total lipid was the sum of the essential lipid and neutral fat.

Specificity of analytical methods

The specificity of a micromethod, when it is usually impossible to characterize fully the substance to be estimated, is frequently far from absolute. Although we feel that, for the most part, we have estimated the substance for which each method was designed, it is important that possible deficiencies of the methods should not be forgotten. For instance, any substance, other than the known cerebrosides, that is soluble in the solvents used and from which reducing substances are released on acid hydrolysis, would be estimated as cerebroside. A similar qualification applies to the method for cholesterol. Although this method can now be described as standard, it is possible that small quantities of other digitonin-precipitable substances that give the Liebermann-Burchard colour reaction would be determined as cholesterol.

The method for total phospholipin is also standard, but that for monoaminophospholipin is less so. In this method known monoaminophospholipins, such as lecithin (phosphatidyl choline) and the kephalins (phosphatidyl ethanolamine, phosphatidyl serine and brain phosphoinositide), would be measured together with any other unknown easily hydrolysed phospholipins. If such substances contained choline in a form readily liberated on hydrolysis, they would be estimated as lecithin; if, as seems more likely, such unknown phospholipins contained no easily hydrolysed choline, they would be estimated as kephalin. In degenerating nerve, however, it is possible that choline-containing degradation products of either sphingomyelin or lecithin may be present. If such a substance were both soluble in light petroleum and insoluble in acetone, it would be estimated as lecithin. One possible degradation product of lecithin, β-glycerylphosphorylcholine, studied by Schmidt, Hershman & Thannhauser (1945), is insoluble in light petroleum, and so would not be present in the phospholipin extracts. Since sphingomyelin is measured as the difference between the total P and the readily hydrolysed P of the

* See, however, Chibnall, Piper & Williams (1936), who found that lignoceric acid from the brain cerebroside, kerasin, was a mixture of *n*-heneicosane-, *n*-tricosane- and *n*-pentacosane-1-carboxylic acids, whilst phrenosinic acid from the brain cerebroside, phrenosin, was a mixture of 2-hydroxy-*n*-heneicosane-, -*n*-tricosane- and -*n*-pentacosane-1-carboxylic acids.

lipid extract, unknown phospholipins, not easily hydrolysed, would be determined as sphingomyelin.

The method for total fatty acid is also not specific, and, in addition to cholesterol, for which correction is made, other non-saponifiable lipids may be measured. The sphingolipids are difficult to saponify and, even if saponification is complete, part of the liberated sphingosine may pass into the light petroleum together with the fatty acids and cholesterol. This may account for the difference in the figures for neutral fat calculated from neutral-fat fatty acid and those calculated from glycerol (some 30% for the control nerves). Also the glycerol of any degradation product of either triglyceride or phosphoglyceride that is soluble in light petroleum and not precipitated by acetone would be measured by the methods used.

Accuracy of analytical methods

The coefficient of variation of the overall procedure for each of the individual lipids was as follows: cerebroside ±3·5%, free cholesterol ±0·9%, total cholesterol ±0·9%, total phospholipin ±1·3%, monoaminophospholipin ±1·6%, lecithin ±5·1%, total fatty acid ±3·1%, and neutral fat ±3·1%. The accuracy of the methods for those lipids derived by difference would usually be less. Since the mean of duplicate estimations was always recorded, the probable error of each observation was less than the above figure.

Recording of results

For the first 32 days after the operation the wet weight of the degenerating nerve was greater than that of the same length of nerve from the control side. This presented the problem of how best to record the results. To express the results in terms of unit wet weight of the degenerating nerve would be misleading, while to express them in terms of unit dry weight of the degenerating nerve would also be unsatisfactory, for the degenerating nerve contained much less lipid than the normal nerve, and lipid accounts for a high percentage of the dry weight of nerve. Therefore we have expressed all results for degenerated nerve in terms of the wet weight of the same length of control nerve from the opposite side. This is equivalent to the fresh weight of the degenerating nerve before it was sectioned, i.e. at zero time.

RESULTS

Animals were killed at intervals of 4, 8, 16, 32, 64 and 96 days after the nerve section. Table 1 gives the mean and the standard error of the mean for the concentration of cerebroside, total, free and ester cholesterol, total phospholipin and essential lipid in the control and degenerated nerves. The table also gives the value of P obtained for each lipid in testing the significance of the difference between the means for the degenerated and control nerves.

The concentration of cerebroside, 2·4 mg./100 mg. for the control nerves, changed little during the first 8 days of degeneration and then decreased rapidly, reaching 0·6 mg./100 mg., or 25% of the total, by 32 days. The concentration of cerebroside then decreased more gradually, no measurable cerebroside remaining by 96 days.

Table 1. *Lipids of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve.)

	Control		Degenerated (4 days)			Degenerated (8 days)			Degenerated (16 days)		
No. of animals ...	30		5			7			5		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Cerebroside	2·40	±0·11	2·48	±0·32	>0·7	2·30	±0·23	>0·6	1·32	±0·12	<0·01
Total cholesterol	3·21	±0·05	3·22	±0·16	>0·9	3·09	±0·15	>0·3	2·66	±0·19	<0·01
Free cholesterol	3·19	±0·05	3·17	±0·16	>0·8	2·92	±0·13	<0·05	1·66	±0·06	<0·01
Ester cholesterol (total – free cholesterol)	0·02	±0·02	0·04	±0·02	>0·2	0·17	±0·04	<0·01	1·00	±0·17	<0·01
Total phospholipin	6·04	±0·13	6·12	±0·26	>0·8	6·00	±0·55	>0·9	2·98	±0·13	<0·01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11·63	±0·26	11·82	±0·56	>0·3	11·43	±0·87	>0·7	6·96	±0·29	<0·01

	Control		Degenerated (32 days)			Degenerated (64 days)			Degenerated (96 days)		
No. of animals ...	30		5			5			3		
	Mean	S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Cerebroside	2·40	±0·11	0·60	±0·16	<0·01	0·48	±0·11	<0·01	0	±0	<0·01
Total cholesterol	3·21	±0·05	1·94	±0·19	<0·01	1·02	±0·05	<0·01	0·77	±0·05	<0·01
Free cholesterol	3·19	±0·05	1·15	±0·05	<0·01	0·54	±0·06	<0·01	0·29	±0·05	<0·01
Ester cholesterol (Total – free cholesterol)	0·02	±0·02	0·79	±0·35	<0·01	0·48	±0·07	<0·01	0·47	±0·08	<0·01
Total phospholipin	6·04	±0·13	1·30	±0·13	<0·01	0·74	±0·05	<0·01	0·60	±0	<0·01
Essential lipid (cerebroside + total cholesterol + total phospholipin)	11·63	±0·26	3·86	±0·26	<0·01	2·22	±0·14	<0·01	1·37	±0·04	<0·01

The concentration of total cholesterol, 3·21 mg./100 mg. in the control nerves, changed little during the first 8 days and then decreased steadily during the course of the degeneration, 0·77 mg./100 mg. still remaining after 96 days. On the other hand, free cholesterol, which accounted for almost all of the cholesterol of the control nerves, decreased to 2·92 mg./100 mg. ($P < 0·05$) after 8 days. From 8 to 32 days the concentration of free cholesterol fell rapidly, and thereafter more slowly, only 0·29 mg./100 mg. remaining after 96 days. There was virtually no ester cholesterol in the control nerves and during the period 0–8 days the concentration increased slowly, reaching 0·17 mg./100 mg. ($P < 0·01$) by the 8th day. The concentration of ester cholesterol then increased rapidly, reaching a maximum of 1·0 mg./100 mg. in 16 days. After 16 days the concentration decreased, but more slowly than the concentration of free cholesterol, so that by 96 days more of the cholesterol was in the ester form than in the free.

The concentration of total phospholipin, which was 6·04 mg./100 mg. for the control nerves, also changed little during the first 8 days. It then decreased rapidly between 8 and 32 days and thereafter more slowly, only 0·6 mg./100 mg., or 10 % of the total, remaining after 96 days. The concentration of essential lipid, representing the sum of the concentrations of cerebroside, total cholesterol and total phospholipin, was 11·63 mg./100 mg. in the control nerves. This decreased in a manner similar to the total phospholipin, only somewhat more slowly. By 96 days 1·37 mg./100 mg. essential lipid remained, or 12 % of the control value.

Table 2 gives the figures for the individual phospholipins, viz. lecithin, sphingomyelin and kephalin, and also those for monoaminophospholipin, total phospholipin and myelin lipid. The concentration of none of these substances changed appreciably during the first 8 days. Between 8 and 32 days the concentration of total phospholipin, sphingomyelin and myelin lipid decreased rapidly and after 32 days more slowly, 10, 7 and 6 % respectively remaining after 96 days. Kephalin, the concentration of which was 2·97 mg./100 mg. in the control nerves, disappeared very rapidly between 8 and 32 days, 0·28 mg./100 mg., or only 9 % of the total, remaining at the end of this time. Subsequently, the concentration of kephalin did not change greatly. Lecithin, which

Table 2. *Phospholipins of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve)

		Degenerated							
	Control	(4 days)			(8 days)			(16 days)		
No. of animals ...	30	5			7			5		
	Mean S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total phospholipin	6·04 ±0·13	6·12	±0·26	>0·8	6·00	±0·55	>0·9	2·98	±0·13	<0·01
Monoaminophospholipin	3·70 ±0·14	4·08	±0·43	>0·2	3·84	±0·28	>0·6	1·60	±0·12	<0·01
Lecithin	0·73 ±0·04	0·80	±0·05	>0·4	0·82	±0·06	>0·2	0·51	±0·10	<0·05
Sphingomyelin (Total phospholipin – monoaminophospholipin)	2·33 ±0·12	2·00	±0·39	>0·3	2·19	±0·40	>0·6	1·36	±0·12	<0·01
Kephalin (Monoaminophospholipin – lecithin)	2·97 ±0·13	3·32	±0·46	>0·3	3·01	±0·26	>0·8	1·12	±0·15	<0·01
Myelin lipid (Cerebroside + free cholesterol + sphingomyelin)	7·91 ±0·21	7·72	±0·69	>0·7	7·43	±0·70	>0·3	4·34	±0·16	<0·01

		Degenerated							
	Control	(32 days)			(64 days)			(96 days)		
No. of animals ...	30	5			5			3		
	Mean S.E.M.	Mean	S.E.M.	P	Mean	S.E.M.	P	Mean	S.E.M.	P
Total phospholipin	6·04 ±0·13	1·30	±0·13	<0·01	0·74	±0·05	<0·01	0·60	±0	<0·01
Monoaminophospholipin	3·70 ±0·14	0·66	±0·09	<0·01	0·48	±0·04	<0·01	0·40	±0	<0·01
Lecithin	0·73 ±0·04	0·36	±0·03	<0·01	0·24	±0·03	<0·01	0·18	±0·03	<0·01
Sphingomyelin (Total phospholipin – monoaminophospholipin)	2·33 ±0·12	0·66	±0·13	<0·01	0·24	±0·07	<0·01	0·17	±0·04	<0·01
Kephalin (Monoaminophospholipin – lecithin)	2·97 ±0·13	0·28	±0·08	<0·01	0·24	±0·02	<0·01	0·23	±0·04	<0·01
Myelin lipid (Cerebroside + free cholesterol + sphingomyelin)	7·91 ±0·21	2·40	±0·27	<0·01	1·28	±0·17	<0·01	0·50	±0·06	<0·01

was present to the extent of only 0·73 mg./100 mg. in the control nerves, decreased less rapidly than either the total phospholipin or myelin lipid.

Table 3 gives the figures for total fatty acid, neutral fat calculated from glycerol, neutral fat calculated from neutral-fat fatty acid, and total lipid.

Table 3. *Fatty acids and neutral fat of cat nerve during Wallerian degeneration*

(mg./100 mg. wet wt. of control nerve. No. of animals is stated in parentheses under each result.)

	Control		Degenerated (4 days)			Degenerated (8 days)			Degenerated (16 days)		
	Mean	S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Total fatty acid	14·54 (20)	±0·50	10·30 (3)	±1·13	<0·01	11·26 (5)	±1·00	<0·01	10·48 (4)	±0·83	<0·01
Neutral fat (calculated from glycerol)	6·91 (19)	±0·44	4·41 (4)	±0·76	<0·05	3·65 (4)	±0·27	<0·01	6·22 (3)	±0·88	>0·5
Neutral fat (calculated from neutral-fat fatty acid)	10·14 (20)	±0·60	5·24 (3)	±1·00	<0·01	6·60 (5)	±0·59	<0·01	7·78 (4)	±0·94	>0·1
Total lipid (Essential lipid + neutral fat)	18·36 (19)	±0·40	16·00 (4)	±1·11	<0·05	14·52 (4)	±1·65	<0·01	12·80 (3)	±1·09	<0·01

	Control		Degenerated (32 days)			Degenerated (64 days)			Degenerated (96 days)		
	Mean	S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Total fatty acid	14·54 (20)	±0·50	12·05 (2)	±2·86	>0·1	10·57 (3)	±0·54	<0·01	10·37 (3)	±2·33	<0·02
Neutral fat (calculated from glycerol)	6·91 (19)	±0·44	7·49 (2)	±3·97	>0·7	9·58 (3)	±0·57	<0·05	6·61 (2)	±1·69	>0·8
Neutral fat (calculated from neutral-fat fatty acid)	10·14 (20)	±0·60	11·02 (2)	±2·94	>0·6	9·85 (3)	±0·58	>0·8	10·10 (3)	±2·37	>0·9
Total lipid (Essential lipid + neutral fat)	18·36 (19)	±0·40	11·35 (2)	±3·35	<0·01	11·87 (3)	±0·59	<0·01	7·95 (2)	±1·75	<0·01

The concentration of total fatty acid fell from 14·54 mg./100 mg. to 10·30 mg./100 mg. in 4 days ($P < 0·01$) and remained close to this figure throughout the course of the degeneration. For each of the periods studied the difference between the values for the degenerating and control nerves was statistically significant, except for the 32-day nerves where fatty-acid figures were available for two animals only. It is likely that, had the group been larger, this figure also would have been significant.

The concentration of neutral fat estimated as glycerol was 6·91 mg./100 mg. in the control nerves. This fell to 4·41 mg./100 mg. after 4 days ($P < 0·05$) and 3·65 mg./100 mg. after 8 days ($P < 0·01$). The value for neutral fat calculated from neutral-fat fatty acid was 10·14 mg./100 mg. for the control nerves, falling to 5·24 mg./100 mg. after 4 days ($P < 0·01$) and 6·6 mg./100 mg. after 8 days ($P < 0·01$). After 8 days the concentration of neutral fat determined by either method did not differ significantly from that of the control nerves. There is an obvious discrepancy between the concentration of neutral fat estimated as glycerol and that calculated from neutral-fat fatty acid. Since the figure for neutral-fat fatty acid was derived by difference and includes errors, not only in the total fatty acid procedure, but also those in the estimation of phospholipin, cholesterol and cerebroside, it can be considered as little more than an approximation. It is reassuring to note that, despite the differences in the values for neutral fat obtained by the two methods, the general trend of the figures was the same for each. Both methods demonstrated the initial fall in the concentration of neutral fat after 4 and 8 days, followed by a return to normal values.

The concentration of total lipid was 18·36 mg./100 mg. for the control nerve and decreased steadily throughout the course of the degeneration, reaching 7·95 mg./100 mg., or 43 % of the total, after 96 days.

In Table 4 the figures for each lipid are expressed as a percentage of the figure for the control nerve of the opposite side, except for ester cholesterol where the figure is expressed as a percentage of the highest value, that of the 16th day. By presenting the data from Tables 1–3 in this manner it is possible to appreciate the rate at which each lipid disappears.

Table 4 also shows the wet weight of the degenerating nerve expressed as a percentage of the weight of the control nerve. The wet weight was greatest after 4 days ($P < 0·01$), but the figures for the wet

Table 4. *Lipids of cat nerve during Wallerian degeneration*

(Concentrations expressed as a percentage of the control nerve concentration, except for ester cholesterol)

	Control	Degenerated					
		4 days	8 days	16 days	32 days	64 days	96 days
Cerebroside	100	103	96	55	25	20	0
Total cholesterol	100	100	96	83	60	32	24
Free cholesterol	100	99	92	52	36	17	9
Ester cholesterol (expressed as a percentage of 16-day concentration)	2	4	17	100	79	48	47
Total phospholipin	100	101	99	49	22	12	10
Monoaminophospholipin	100	110	104	43	18	13	11
Lecithin	100	110	112	70	49	33	25
Sphingomyelin	100	86	94	58	28	10	7
Kephalin	100	112	101	38	9	8	8
Essential lipid	100	102	98	60	33	19	12
Myelin lipid	100	98	94	55	30	16	6
Total fatty acid	100	71	77	72	83	73	71
Neutral fat (calculated from glycerol)	100	64	53	90	108	139	96
Neutral fat (calculated from neutral-fat fatty acid)	100	52	65	79	109	97	100
Total lipid	100	87	79	70	62	65	43
Wet weight	100	134	122	126	120	105	90

weight after 8 ($P<0{\cdot}01$), 16 ($P<0{\cdot}02$) and 32 days ($P<0{\cdot}05$) were all significantly greater than those of the control nerves. After 32 days the difference between the wet weight of the degenerating and the control nerves was not statistically significant.

DISCUSSION

Previous observations. The increase in the wet weight of degenerating nerve was reported by Mott & Halliburton (1901*a*, *b*) and confirmed by May (1930) and Abercrombie & Johnson (1946*b*).

Our findings on the changes in the concentration of lipids agree well with those of previous workers. Noll (1899) reported a decrease in the concentration of 'protagon' in degenerating horse and dog nerves. It is unfortunate that this excellent work was done under the shadow of the 'protagon' controversy. There can be no doubt that 'protagon', once believed to be the mother of all brain substances, contributed greatly to our present ignorance of the chemistry of 'myelin'. Excellent accounts of this interesting controversy are those of Posner & Gies (1905) and MacLean (1918).

Noll (1899) estimated 'protagon' in nerves by the reducing substances liberated during acid hydrolysis. For the most part, he was measuring galactose liberated from the cerebroside. He reported that the total solid of an ethanol extract of a dog nerve, which had degenerated for 15 days, decreased less than the phosphorus of the extract, i.e. that the phospholipin disappeared from the nerve more rapidly than the total lipid. He also reported that 'protagon', probably cerebroside, of another nerve, which had degenerated for 16 days, decreased to 54 % of the value for the control nerve. This result can be compared with our mean cerebroside concentration of 55 % for 16-day cat nerves.

Mott & Halliburton (1901*a*, *b*) reported that the concentration of total phosphorus in a degenerating cat nerve fell to zero in 29 days. The method for phosphorus estimation must have been extremely insensitive, for our experiments indicate that considerable phospholipin remains in the nerve after 32 days. May (1930) also found a decrease in total phosphorus in degenerating rabbit nerves, greatest between the 7th and 49th day. There was still 35 % of the total phosphorus present even after 100 days. He also reported a rapid decrease of lipid P after 7 days and a considerable increase in water-soluble P throughout the whole period of the degeneration.

The composition of 'myelin'. While studying the chemical nature of the lipids of 'myelin', Johnson *et al.* (1948*b*) found that the distribution of lipids in peripheral nerve resembled that of the lipids of the white matter of the brain rather than that of the grey matter. Brain white matter and peripheral nerve, both of which are rich in myelinated fibres, differed from brain grey matter in that they contained more cerebroside, free cholesterol and sphingomyelin (Johnson *et al.* 1948*a*). Subsequently, Johnson *et al.* (1949*a*) reported that it was these same three lipids, cerebroside, free cholesterol and sphingomyelin, that distinguished the white matter of the adult brain from that of the brain of the newborn infant, where myelination is incomplete. It was suggested that these three lipids, i.e. free cholesterol and the two sphingolipids, cerebroside (or glycosphingoside) and sphingomyelin (or phosphosphingoside), rather than the phosphoglycerides, lecithin and kephalin, were the principal lipid components of the myelin sheath.

These lipids were called the myelin lipids. Carter, Haines, Ledyard & Norris (1947) found a high concentration of the sphingolipids, cerebroside and sphingomyelin, in ox spinal cord.

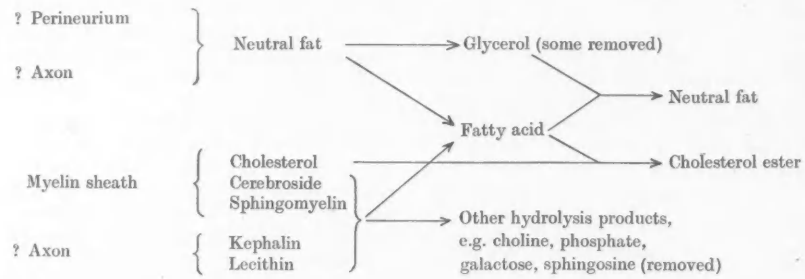
Each of the myelin lipids decreased in the degenerating nerve at approximately the same rate and to the same extent (Table 4). The change in the concentration of none of the other lipids resembled that of the myelin lipids; total lipid decreased much less rapidly; neutral fat decreased early, and then returned to normal; total cholesterol decreased more slowly; cholesterol ester, not present in normal nerve, appeared during the course of the degeneration; total phospholipin decreased at first more rapidly and then more slowly than the myelin lipid; lecithin decreased much more slowly and kephalin more rapidly (Table 7). The finding that cerebroside, free cholesterol and sphingomyelin decreased at the same rate and to the same extent is additional evidence supporting our previous suggestion that these substances, rather than lecithin and kephalin, are the principal lipid constituents of the myelin sheath.

Frequently the essential lipids are regarded as the important structural lipids of the body. Because a large proportion of the essential lipid of nerve is myelin lipid, they both tend to disappear from a degenerating nerve at a similar rate (Table 4). Although the essential lipids are important elements of the central nervous system as a whole, the myelin lipids would appear to play a more important role in the structure of the myelin sheath.

Abercrombie & Johnson (1946*b*) found that most of the non-extractable nitrogen of rabbit nerve disappeared at a time similar to that during which we have observed the disappearance of the myelin lipid in cat nerve. This non-extractable nitrogen probably represents the so-called 'neurokeratin' (Block, 1937) and may be an important structural element of the myelin sheath.

Hitherto most of our knowledge of the constitution of the myelin sheath has come from polarized light and X-ray diffraction studies (Schmitt & Bear, 1939; Schmitt, Bear & Palmer, 1941). These studies indicate that the myelin sheath consists of coaxial concentric sheets of oriented lipid molecules alternating with thin, possibly unimolecular, layers of protein. The sheath shows a characteristic birefringence with the optical axes directed radially and a definite X-ray diffraction pattern. Such valuable studies tell us little, however, of the chemical nature of the lipid in this lipid-protein complex. It is suggested that this complex is rich in free cholesterol and the sphingosine-containing lipids, cerebroside and sphingomyelin.

Lipids of myelin during Wallerian degeneration. We can now give an outline of some of the changes that occur in the lipids when a peripheral nerve undergoes Wallerian degeneration. During the early stages the water content of the nerve increases, the absolute amount of the neutral fat decreases and there is little change in the myelin lipids. From 8 to 32 days there is a steady decrease in the concentration of the myelin lipids, i.e. cerebroside, free cholesterol and sphingomyelin. These substances presumably are slowly hydrolysed and the products of hydrolysis, e.g. glycerol, fatty acid, choline, galactose, sphingosine and phosphate, are removed. There is, in addition, a rapid hydrolysis of kephalin and a slower hydrolysis of lecithin. Some of the fatty acids, liberated during the hydrolysis, may combine with free cholesterol to form cholesterol ester and others may be converted into neutral fat. These changes can be represented schematically as follows:



It should be stressed that the analyses were performed on the whole nerve and therefore represent not only the lipids of myelin, but also those of the axon, Schwann cells, macrophages, perineural and endoneural connective tissue and, perhaps, some epineural connective tissue also. The neutral fat is probably chiefly in the connective tissue, but some may be in the axon. The high proportion of kephalin and lecithin in grey matter of brain (Johnson *et al.* 1948*a*, 1949*a*) would suggest that these lipids are chiefly constituents of the axon.

A scheme, admittedly hypothetical, representing the demyelination process was given by Page (1937). The chief experimental evidence, quoted in support of the scheme, was the autolysis experiments of Jungmann & Kimmelstiel (1929) and Backlin (1930). The former workers found that cerebroside decreased and inorganic phosphorus increased when rabbit whole brain stood in either oxygen or nitrogen. Backlin (1930) described a fall in the concentration of free cholesterol and cerebroside, and a possible increase in the concentration of phospholipin when a rabbit brain stood for 24 hr. However, he presented the results of one experiment only. Johnson, McNabb & Rossiter (1949*c*) found no change in the concentration of cerebroside or total cholesterol in slices of cat brain which were incubated in a buffer for periods of time up to 14 days.

The text-book description of the chemistry of demyelination, that 'myelin' is composed of phospholipin which is converted into triglyceride, is probably derived from the views of Mott & Halliburton (1901*a*, *b*). That phospholipin is converted into triglyceride may be true in part, but phospholipin hydrolysis and triglyceride formation are only two of a number of changes that occur. In the past, undue emphasis has been placed on these two processes (Mott & Barratt, 1899; Setterfield & Sutton, 1935).

The period of greatest destruction of 'myelin' (8–32 days) is characterized by a decrease in the concentration of the myelin lipids, an increase in the concentration of ester cholesterol, and a return to normal of the concentration of neutral fat. During this time macrophages appear along the length of the degenerating nerve and there is a great proliferation of the Schwann cells (Holmes & Young, 1942; Young, 1942; Abercrombie & Johnson, 1946*a*). It is possible that either the macrophages or the Schwann cells contribute enzymes to aid the degradation of the lipids. The histological evidence suggests that quite large pieces of organized 'myelin' are engulfed by the macrophages and subsequently degraded. The 'myelin' is most likely destroyed while the macrophages are in the degenerating nerve, although the possibility that the macrophages remove themselves and destroy the 'myelin' elsewhere cannot be excluded. The remarkable similarity of the disappearance curves for each of the individual myelin lipids would favour the theory of particulate ingestion followed by rapid hydrolysis or removal. Johnson *et al.* (1949*c*) showed that, when brain tissue was autolysed *in vitro*, phospholipin, chiefly kephalin and sphingomyelin, was destroyed, but that there was no destruction of cerebroside or cholesterol. This would indicate that the *in vitro* degradation of the myelin lipids is different from their *in vivo* degradation. Possibly enzymes, other than those present in the non-degenerating nervous system, e.g. those of the macrophages or the Schwann cells, are necessary for complete breakdown of the myelin lipids.

Our findings are also of interest because the quantitative changes in the concentration of lipids in a degenerating nerve can be correlated with the results of previous physiological and biological investigations of Wallerian degeneration. A discussion of our results in the light of such studies will have to be postponed for a subsequent paper.

SUMMARY

1. The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin, total fatty acid and neutral fat was determined in degenerating cat sciatic nerves at intervals of time from 4 to 96 days after nerve section. From these figures the concentration of ester cholesterol, sphingomyelin, kephalin, essential lipid, myelin lipid, and total lipid was calculated.

2. The wet weight of the degenerating nerve increased rapidly, reaching a maximum in 4 days and returning to normal after 64 days.

3. The total lipid content of the nerve decreased steadily throughout the course of the degeneration.

4. Neutral fat decreased rapidly, reaching a minimum between 4 and 8 days, and returned to normal by 32 days.

5. The myelin lipids (cerebroside, free cholesterol and sphingomyelin) changed little during the first 8 days and then decreased rapidly, and to the same extent, between 8 and 32 days.

6. Cholesterol changed little during the first 8 days. Between 8 and 32 days free cholesterol decreased rapidly and ester cholesterol, absent in control nerves, increased, reaching a maximum by 16 days.

7. Total phospholipin, which changed little during the first 8 days, decreased rapidly between 8 and 32 days. Sphingomyelin decreased at a similar rate, whereas kephalin decreased more rapidly and lecithin more slowly.

8. The results are discussed with reference to the chemical nature of the lipids of 'myelin' and to the changes that occur in the lipids when a nerve undergoes Wallerian degeneration.

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Determination of *p*-Aminosalicylic Acid as *m*-Aminophenol

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Several methods have been described for the estimation of *p*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid) in blood (Lehmann, 1946; Ragaz, 1948; Tennent & Leland, 1948, 1949; Klyne & Newhouse, 1948). None of the procedures, developed in connexion with therapeutic trials of this drug in tuberculosis, were micromethods; our aim was to develop a method suitable for use with capillary blood. Bratton & Marshall's (1939) estimation of sulphonamides in blood seemed an obvious choice. Such a method was used (but not described) by McClosky, Smith & Frias (1948). These workers recorded erratic results with *p*-aminosalicylic acid, but obtained quantitative linear curves by allowing solutions of the drug to stand for several days at room temperature, or by heating them at 100° for 30–60 min.

Aqueous solutions of *p*-aminosalicylic acid are known to be decarboxylated on standing or heating (Venkataraman, Venkataraman & Lewis, 1948; Rosdahl, 1948; Oberweger, Seymour & Simmonite, 1948). The findings of McClosky *et al.* (1948) could be explained as the decarboxylation of *p*-aminosalicylate followed by diazotization of *m*-aminophenol. We thought that such a technique could be used for the micro-estimation of *p*-aminosalicylic acid and set about establishing conditions for complete decarboxylation.

While this work was in progress four diazotization methods were described. One is an adaptation by Marshall (1948) of the original Bratton & Marshall (1939) procedure, on which the two other methods (Way, Smith, Howie, Weiss & Swanson, 1948; Newhouse & Klyne, 1949) are also based. In the fourth method (Desbordes & Henry, 1948) diazotized *p*-aminosalicylic acid is coupled with thymol.

EXPERIMENTAL

Bratton & Marshall's procedure for estimating total sulphonamide in blood was progressively modified, and two alternative methods, *A* and *B*, were finally adopted.

Reagents. Trichloroacetic acid (20% w/v in water). H_2SO_4 (21·5±0·5N; made by adding 60 ml. conc. H_2SO_4 to 40 ml. water, titrating and readjusting the volume). NaOH (8·1N). $NaNO_2$ (1% w/v in water). Ammonium sulphamate (5% w/v in water). *N*-1-Naphthylethylenediamine dihydrochloride (0·1% in water). Ethanol (95%) or industrial methylated spirit.

Standard solutions. (1) Stock standard equivalent to 100 mg. *p*-aminosalicylic acid/100 ml.: 137·9 mg. pure sodium *p*-aminosalicylate dihydrate is dissolved in 100 ml. water; or 71·2 mg. pure *m*-aminophenol is dissolved in 100 ml. 0·01N-HCl. These can be kept in colourless bottles at room temperature for at least 8 weeks. (2) Dilute standards representing 5, 10 and 20 mg. *p*-aminosalicylic acid/100 ml. blood: 0·25, 0·50 and 1·0 ml. of solution (1) and 15 ml. 20% (w/v) trichloroacetic acid are diluted to 100 ml. with water. These are referred to as '5, 10 and 20 mg. solutions'. With standard solutions containing *p*-aminosalicylate, 2·0 ml. samples are treated as supernatant liquids of the test solutions; using *m*-aminophenol standards, heating may be omitted.

Method A

Oxalated or freshly drawn capillary blood (0·2 ml.) is added to water (3·2 ml.), and the tube is allowed to stand for 30 min. Trichloroacetic acid (0·6 ml.) is added, and the mixture is shaken and centrifuged. Supernatant liquid (2·0 ml.), transferred to a centrifuge tube graduated at 4 ml., is heated with H_2SO_4 (0·5 ml.) in a boiling water bath for 1 hr. The solution is cooled in tap water (approx. 15°), partially neutralized with NaOH (1·0 ml.) and again cooled. Water is added to adjust the volume to 4·0 ml.

$NaNO_2$ solution (0·2 ml.) is added, and the solution is shaken. After 5 min. ammonium sulphamate solution is added, followed 20±1 sec. later by naphthylethylenediamine (1·0 ml.), the tube being shaken after each addition.

The pink colour is allowed to develop for at least 2·5 hr. and is then compared with the appropriate standard solution. An Evans Electroselenium Ltd. portable photoelectric colorimeter with an Ilford 625 filter gives readings proportional to concentration if set to zero against a full reagent blank (2 ml. 3% w/v trichloroacetic acid taking the place of the supernatant liquid). An Ilford 404 green filter, though not giving fully linear results, has also been found satisfactory if a calibration curve is prepared using all three standards (the 5, 10 and 20 mg. solutions).

Method B

Method *A* is carried out to the coupling stage. 20±1 sec. after coupling with naphthylethylenediamine solution, ethanol (1·0 ml.) is added, the tubes are shaken, and the colours compared after 1 hr. These are proportional to concentration when using an Ilford 625 filter and a modified reagent blank (2·0 ml. water taking the place of the supernatant liquid). The Ilford 404 filter is unsuitable for this method.

RESULTS

Decarboxylation and hydrolysis. Our first experiments showed that solutions of *p*-aminosalicylate heated with concentrated hydrochloric acid gave deeper colours than solutions treated with hydrochloric acid in the cold or heated with trichloroacetic acid. Sulphuric acid was substituted for hydrochloric to prevent the formation of hydroxyazo compounds during the diazotization stage. The strength of acid and the heating time finally adopted give complete decarboxylation, as is shown by carrying out the method on *p*-aminosalicylate and *m*-aminophenol solutions with and without heating (Table 1). Determinations carried out on 20 mg.

Table 1. *The conversion of* p-*aminosalicylate into* m-*aminophenol*

(20 mg. standards used in each case.)

Exp. no.	Compound and treatment	Final concentration of *m*-aminophenol expressed as: Colorimeter reading ($100 \times E$)	Final concentration of *m*-aminophenol expressed as: Percentage of result in Exp. 1
1	*m*-Aminophenol, not heated	54	100
2	*m*-Aminophenol, heated	55·5	103
3	*p*-Aminosalicylate, not heated	17	31·5
4	*p*-Aminosalicylate, heated	53	98

solutions of *m*-aminophenol and *p*-acetamidosalicylic acid show that the *N*-acetyl derivative of *p*-aminosalicylic acid is completely hydrolysed by this treatment. Thus the present estimation is one of 'total *p*-aminosalicylate'. When heating is omitted, solutions of the *N*-acetyl compound give no colour reaction.

Diazotization. Partial neutralization with sodium hydroxide gives the moderately acid medium usual for diazo coupling. Observations which led to this step being introduced are summarized in Table 2. Diazotization for 5 min. was chosen as a convenient time, since the colour intensity/diazotization time curve showed only a very gradual decrease between 3 and 10 min. (Colorimeter reading at 3 min., 60; 5 min., 59·5; 10 min., 59; 15 min., 57.) Minor changes in the temperature of diazotization introduce appreciable errors; all test and standard solutions should be placed in the same cooling bath.

Table 2. *Effect of* pH *on colour intensity in the estimation of* p-*aminosalicylate as* m-*aminophenol*

(20 mg. standards used in each case.)

Exp. no.	Decarboxylation medium	Diazotizing and coupling medium	Colour intensity (percentage of value in Exp. 2)
1	Strongly acid (21·5 N-H_2SO_4)	Strongly acid (unchanged)	51·5
2	Strongly acid (21·5 N-H_2SO_4)	Less acid (partly neutralized with 8·1 N-NaOH)	100
3	Less acid (9 N-H_2SO_4)	Less acid (unchanged)	81
4	Less acid (9 N-H_2SO_4)	Strongly acid (9 N-H_2SO_4 added until pH approx. that of Exp. 1)	12

Newhouse & Klyne (1949) allowed 10 sec. between adding ammonium sulphamate and coupling. Our sulphamate time of 20 sec. was adopted directly from their method. It gives colours about 20% deeper than we had formerly obtained using longer times (cf. Table 3).

Table 3. *The effect of varying diazotizing and sulphamate times on the colour intensities obtained from* p-*aminosalicylate and* m-*aminophenol solutions*

(20 mg. standards used in each case.)

Exp. no.	Compound	Diazotizing time (min.)	Sulphamate time	Colorimeter reading ($100 \times E$)
1	*p*-Aminosalicylate	5	20 sec.	57
2	,,	5	30 min.	38
3	,,	20	20 sec.	49
4	,,	20	10 min.	44·5
5	*m*-Aminophenol	5	20 sec.	58
6	,,	5	20 min.	50
7	,,	20	20 sec.	53
8	,,	20	20 min.	43

Properties of the dye solutions. The colours given by methods *A* and *B* follow Beer's law between 2·5 and 20 mg./100 ml. levels using an Ilford 625 filter. The similar development (Fig. 1) of the colours obtained from equimolar solutions of *p*-aminosalicylate after heating and of *m*-aminophenol confirms the identity of the two dyes. Unheated solutions of *p*-aminosalicylate develop colour more rapidly than either of these two solutions. This indicates a structural difference between the dyes rather than a partial decarboxylation of the acid in the cold which would then give less of the same dye.

Both conclusions are borne out by the absorption spectra of the dyes (Fig. 2). These were recorded by Dr I. D. P. Wootton, using a Beckmann spectrophotometer. The solutions used were those obtained by carrying through 20 mg. solutions of *p*-aminosalicylate and *m*-aminophenol. The heated *p*-aminosalicylate and the unheated *m*-aminophenol solutions have identical spectra. The unheated *p*-aminosalicylate solution gives a dye with the expected lower extinction values and an absorption maximum at a somewhat shorter wavelength.

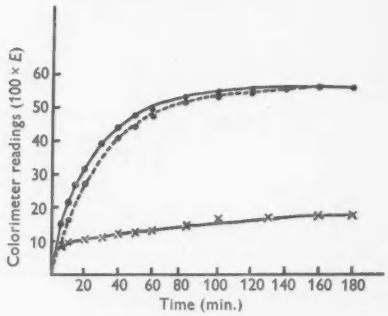

Fig. 1. Rate of colour development in the estimation of *p*-aminosalicylate and *m*-aminophenol (20 mg. standards, using method *A*): - - - - - -, *m*-aminophenol, unheated; ————, *p*-aminosalicylate, heated; —×—×—×, *p*-aminosalicylate, unheated.

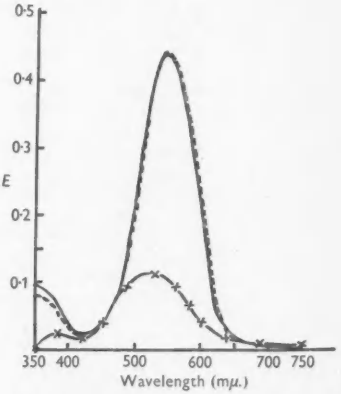

Fig. 2. Absorption spectra of dyes (20 mg. standards, using method *A*): - - - - - -, *m*-aminophenol, unheated; ————, *p*-aminosalicylate, heated; —×—×—×, *p*-aminosalicylate, unheated.

The colours produced in method *A* are stable to light and are unchanged after 90 hr. at room temperature. The solution of the dye obtained from unheated *p*-aminosalicylate is stable only for a few hours. The dye slowly separates as a flocculent precipitate, leaving a colourless solution. On shaking

the mixture the precipitate is dispersed; the solution appears clear and has the same colour intensity as it had before flocculation occurred. The precipitate can be separated easily by filtration. Since in the aqueous acid medium containing a high salt concentration the more acid dye produced from the unheated *p*-aminosalicylate would probably be less soluble than its *m*-aminophenol analogue, the precipitate was filtered off and the filtrate kept for some time. No further colour developed; thus the low colour intensity of this solution is not due to a solubility effect leading to incomplete dye formation.

Table 4. *Recoveries of* p-*aminosalicylate added to blood*

(Aqueous *p*-aminosalicylate solution (100 mg./100 ml.) added to oxalated blood.)

Blood *p*-aminosalicylate level (mg./100 ml.)	No. of exp.	Recovery (%)	
		Range	Mean
10	17	89–102	96
20	8	91–102	98

Attempts were also made to follow the diazotization of *p*-aminosalicylic acid and *m*-aminophenol by means of free nitrous acid estimations. The *m*-phenylenediamine method (see Welcher, 1947) used cannot be described as a strictly quantitative method; it does indicate, however, that the amount of nitrous acid used in the reaction is in excess of that required by a simple diazotization.

Recoveries. The results of these are given in Table 4. The determinations were carried out on fresh oxalated blood to which the requisite amount of *p*-aminosalicylate (stock standard solution) was added.

Penicillin did not interfere with the estimation. Sulphonamides and sulphones (Sulphetrone) do, of course, interfere. An unsuccessful attempt was made to overcome this difficulty by converting the diazotized *m*-aminophenol to resorcinol and estimating the product by an adaptation of Seliwanoff's reaction.

SUMMARY

1. A method for the estimation of 'total *p*-aminosalicylate' in capillary blood is described.

2. *p*-Aminosalicylic acid and its *N*-acetyl derivative are quantitatively converted to *m*-aminophenol; this compound gives deeper colours than *p*-aminosalicylate when diazotized and coupled with *N*-1-naphthylethylenediamine.

3. The main aspects of the method have been surveyed and the behaviour of *p*-aminosalicylic acid and *m*-aminophenol compared.

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